THE INOCULUM ECOLOGY OF *BOTRYTIS CINEREA* IN ROOIBOS NURSERIES

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SUMMARY

Grey mould, caused by *Botrytis cinerea*, is the most important foliar disease of rooibos seedlings. Although the disease is primarily controlled with applications of fungicides, the improvement of cultural methods of disease management should lessen this dependence on chemical control. Such improvements would, however, not be possible without knowledge of the inoculum sources and dispersal of the pathogen. The aim of this study was to investigate the inoculum ecology of *B. cinerea* in rooibos nurseries in order to identify primary sources of inoculum and to improve the environmentally friendly management of the disease.

The study was conducted in four nurseries over two production seasons (March to July 2003 and 2004). Levels of airborne inoculum of *B. cinerea* were monitored on a monthly basis inside and around the nurseries with spore traps. Samples of plant material and organic debris were taken in the corresponding areas to determine the incidence of plant material infected by the pathogen and the incidences of grey mould in the nurseries were recorded. Low numbers of *B. cinerea* colonies were observed on the spore traps. Similar levels of airborne inoculum were observed inside and around the nurseries. The incidence of plant material yielding *B. cinerea* was higher outside the nurseries than inside, indicating the importance of such materials as potential sources of inoculum. Since patterns of airborne inoculum observed in this study confirmed reports of the local dispersal of *B. cinerea*, the removal of possible hosts outside the nurseries could aid in the management of grey mould in rooibos nurseries.

Resistance to dicarboximide fungicides is a genetically stable trait in *B. cinerea*, and therefore has the potential to be used as a phenotypic marker. This marker can be used to gain knowledge on the dispersal of *B. cinerea* inoculum inside and outside rooibos nurseries. Isolates of *B. cinerea* collected from the air and from plant material in and around four rooibos nurseries were assessed for resistance to iprodione at 1 and 3 µg/ml a.i. Some of the isolates showed resistance to iprodione at 1 µg/ml a.i. However, none of the isolates showed resistance at 3 µg/ml a.i. iprodione. The initial incidence of dicarboximide-resistance at the nurseries was slightly higher than expected. As the season progressed, the incidence of iprodione-resistant isolates decreased towards May, after which an increase was observed towards July. A relatively high percentage of isolates collected outside the nurseries was found to be

dicarboximide-resistant. Two of the nurseries had a significant higher incidence of resistant isolates on plant material collected inside, than on plant material collected outside the nursery. However, when looking at resistance levels of airborne isolates, no significant differences were found in the incidence of resistant isolates sampled inside and outside the four nurseries. The data indicated the importance of organic debris and seed-borne infections in the survival and dispersal of dicarboximide-resistant isolates of the pathogen.

With the current emphasis on organic agriculture the knowledge gained in this study presents valuable possibilities of improving the cultural management of grey mould in rooibos nurseries.

DIE INOKULUM EKOLOGIE VAN *BOTRYTIS CINEREA* IN ROOIBOS KWEKERYE

OPSOMMING

Vaalvrot, veroorsaak deur *Botrytis cinerea*, is die belangrikste bo-grondse siekte van rooibossaailinge. Alhoewel die beheer van die siekte hoofsaaklik op die gebruik van fungisiede berus, behoort die verbetering van verbouingspraktyke hierdie afhanklikheid van chemiese beheer te verminder. Sulke verbeteringe sal egter slegs moontlik wees indien voldoende kennis van die inokulumbronne en verspreiding van die patogeen beskikbaar is. Die doel van hierdie ondersoek was om die inokulum ekologie van *B. cinerea* in rooibos kwekerye te ondersoek sodat primêre inokulumbronne opgespoor en omgewingsvriendelike siektebestuurspraktyke verbeter kan word.

Die ondersoek is in vier kwekerye oor twee produksie seisoene (Maart tot Julie 2003 en 2004) uitgevoer. Vlakke van luggedraagde inokulum van *B. cinerea* is op ’n maandelikse basis met behulp van spoorvangers binne en buite die kwekerye gemonitor. Monsters van plantmateriaal en organiese materiaal is in ooreenstemmende areas geneem om die voorkoms van *B. cinerea* geïnfekteerde plantmateriaal vas te stel en die voorkoms van vaalvrot in die kwekerye is aangeteken. Min *B. cinerea* kolonies is op die spoorvangers waargeneem. Soortgelyke vlakke van luggedraagde inokulum is binne en buite die kwekerye waargeneem. Die hoër voorkoms van *B. cinerea* geïnfekteerde plantmateriaal buite die kwekerye as binne, dui op die belang van sulke materiaal as potensiële inokulumbronne. Aangesien die patrone van luggedraagde inokulum, soos waargeneem in hierdie ondersoek, ander berigte van *B. cinerea* se beperkte verspreidingsvermoë bevestig, kan die verwydering van moontlike alternatiewe gashere buite die kwekerye die bestuur van die siekte binne die kwekerye verbeter.

Weerstand teen dikarboksimied fungisiede is ’n geneties-stabiele kenmerk in *B. cinerea* en het daarom potensiaal om as ’n fenotipiese merker gebruik te word. Hierdie merker kan gebruik word om kennis aangaande die verspreiding van *B. cinerea* in en om rooibos kwekerye in te samel. *Botrytis cinerea* isolate in lug en op plantmateriaal in en om vier rooibos kwekerye is gedurende 2003 en 2004 versamel. Die isolate is vir weerstandbiedendheid teen iprodioon by konsentrasies van 1 en 3 µg/ml aktiewe bestandeel (a.b.) getoets. Isolate met weerstand teen 1 µg/ml a.b. iprodioon is waargeneem, maar nie teen 3 µg/ml nie. Die

aanvanklike voorkoms van dikarboksimiedweerstand by die kwekerye was hoër as verwag. Hierdie vlak het egter gedaal met die verloop van die seisoen tot in Mei, waarna ’n toename tot in Julie waargeneem is. Die persentasie dikarboksimied-weerstandbiedende isolate buite die kwekerye was relatief hoog. In twee van die kwekerye was die voorkoms van weerstandbiedende isolate op plantmateriaal in die kwekerye betekenisvol hoër as op plantmateriaal buite die kwekerye. Daar was egter geen betekenisvolle verskille in die voorkoms van luggedraagde weerstandbiedende isolate nie, ongeag van die kwekery of posisie. Die data dui op die belang van organiese materiaal en saadgedraagde infeksies in die oorlewing en verspreiding van dikarboksimied-weerstandbiedende isolate van die patogeen.

Met die huidige klem op organiese landbou bied die inligting wat in hierdie ondersoek versamel is moontlike praktyke wat geïmplementeer kan word om die beheer van vaalvrot in kwekerye met behulp van verbouingspraktyke te verbeter.

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

Signature:..……..…… Date:…....……...

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INTRODUCTION

Grey mould, caused by *Botrytis cinerea* Pers. ex Pers., is the most important foliar disease of rooibos seedlings. Currently, the disease is primarily controlled with frequent applications of fungicides. The improvement of cultural methods of disease management should lessen this dependence on chemical control. Such improvements would, however, not be possible without knowledge of the inoculum sources and dispersal of the pathogen. Information regarding the ecology of *B. cinerea* is likely to differ between crop systems. The following review is therefore divided into three parts: (i) rooibos production and grey mould in rooibos nurseries, (ii) the epidemiology and ecology of *B. cinerea* and (iii) the resistance of the pathogen to dicarboximide fungicides and the suitability of this trait in studying the movement of inoculum.

Rooibos

Rooibos [*Aspalathus linearis* (Burm. F.) R. Dahlg.] is a leguminous perennial of the bean family that occurs naturally in the Cedarberg Mountains of the Western Cape province. Native inhabitants of this area have used rooibos as a health supplement for centuries, but it has only recently gained international prominence as a health beverage, rooibos tea. The increased interest in this member of the fynbos biome has sparked international research revealing the value of rooibos as a source of nutritional minerals, vitamins C and E and several flavonoids with antioxidative action (Meyer, 2003). Besides anti-allergy and anti-spasmodic activity (Louw, 2003), extracts from rooibos can also prevent binding of HIV to cells (Nakano *et al*., 1997).

Several attempts have been made at growing rooibos outside its natural habitat, but all have failed (Standley, 1999). Consequently the only areas in the world where rooibos is produced commercially remains the area surrounding the Cedarberg and Olifantsriver Mountains (Gleason, 2003) with Nieuwoudtville as the most northern and Eendekuil the most southern districts (J. Brand, Rooibos Ltd., P.O. Box 64, Clanwilliam, 8135, South Africa, personal communication). Approximately 7500 ha of rooibos are planted annually in this area (J. Brand,

personal communication). The demand for rooibos tea is steadily increasing, especially in foreign countries, causing higher than expected sales in 2002 and 2003 for the largest existing rooibos company, Rooibos Ltd. (Clanwilliam, South Africa). In spite of an all-time highest yield of 9 500 000 kg of rooibos in 2003 a shortage in rooibos is still expected in the near future, causing a recent increase in the cost of rooibos products (M. Bergh, Rooibos Ltd., P.O. Box 64, Clanwilliam, 8135, South Africa, personal communication). Considering that all rooibos cultivation in the world is restricted to such a small area, it is self-evident that any factor that limits the yield of marketable rooibos could have serious economic impacts.

Rooibos production

Rooibos seed is produced singly in small pods that develop from yellow, pea-shaped flowers that cover the plants in October. The small seeds are ejected from the pods as soon as they ripen and need to be sieved from the soil surrounding older plants. Before sowing, the seeds are scarred mechanically to improve germination and treated with fungicides to control damping-off, caused by *Fusarium, Pythium* and *Rhizoctonia* spp. (S.C. Lamprecht, ARC–Plant Protection Research Institute, Private Bag X5017, Stellenbosch, 7599, personal communication). Seeds are planted in February and March either in open seedbeds in nurseries (Figure 1) or, in June and July, directly in the field. Approximately 10 ha of rooibos can eventually be planted from 1 kg of seed (J. Brand, personal communication).

Nursery grown seedlings are fertilized and irrigated regularly until June or July when they reach a height of 100–200 mm, and are subsequently transplanted into the field. In the period from April to September of the following year the top 30 cm of young plants are removed to promote vegetative sprouting. After about 18 months in the field the plants are ready to be harvested for the first time in January to March. Depending on the vegetative growth, plants are harvested once a year at the most and remain economically productive for about 6 years after which they are usually burned to clear the soil (J. Brand, personal communication).

The susceptibility of nursery seedlings and mature plants to pathogens and pests differ. Mature plants are, due to their nutritional value, a target of several insect pests which are usually controlled by pesticide applications when necessary. Pathogens of mature plants are an inferior problem when compared with insect pests and drought. Contrarily, in nurseries several diseases caused by fungi such as *Botrytis*, *Colletotrichum*, *Fusarium*, *Pythium* and *Rhizoctonia* spp. are problematic, while fewer insect pests are found (J. Brand, personal communication).

Grey mould in rooibos nurseries

Botrytis cinerea was first identified as the causal agent of grey mould of rooibos seedlings in 1994 (Lamprecht, 1996). It is the most important foliar disease in rooibos nurseries and can cause losses of up to 80% in some seasons if management practices are not implemented properly.

The pathogen usually attacks the lower parts of the stems and leaves, causing wilt-like symptoms and occasionally death (Figure 2). In warm and dry conditions wilt-symptoms may progress quickly to resemble a kind of shepherd's crook. Although other fungi may cause similar symptoms, seedlings affected by grey mould are easily identified by the presence of conidiophores and conidia of *B. cinerea* on affected plant parts (Figure 3). Due to the high density of seedlings in the nurseries the symptoms are usually noticed on small 'patches' of adjacent seedlings which may already include seedlings that have died.

No studies have been done on the epidemiology of *B. cinerea* in rooibos nurseries. The disease is mainly a problem on older seedlings (2–4 months) (Lamprecht, 1996). Several factors may be involved in this phenomenon. The larger size of older seedlings may increase the density of canopies, resulting in higher humidity around, and less penetration of light and fungicides to the lower plant parts. Not only would such conditions favour the growth of *B. cinerea*, but lower light intensities within the canopy might also predispose plant parts to infection (Zhang *et al.*, 1995). The physiological age of the seedlings may also be important, since the receptivity of tissues toward infection by *B. cinerea* have been shown to vary with age (Hausbeck & Moorman, 1996; Sirjusingh & Tsujita, 1996; Coertze & Holz, 2001). Weeds and organic debris may also play a part in promoting the development of the disease by acting as alternative hosts for the pathogen or as inoculum sources, respectively. Sporulating *B. cinerea* have previously been observed on organic debris in nurseries (S.C. Lamprecht, personal communication). Weeds may also increase the humidity inside the seedlings and in doing so, encourage the development of grey mould.

Currently the management of grey mould in nurseries rely heavily on weekly applications of fungicides. Iprodione and pyrimethanil are used weekly in alternation during the later part of each season. Other management strategies are mostly aimed at reducing the humidity around seedlings, i.e. an east-west row orientation, 10 cm row spacing, and a sowing density of 15 g seeds per meter. Farmers are also advised to irrigate the nurseries in the morning to minimise

the persistence of free water on the plants during the night. The frequent removal of weeds and organic debris inside the nurseries are recommended to minimise inoculum sources in the nurseries. These cultural practices are implemented in accordance with the guidelines provided by Rooibos Ltd. (P.O. Box 64, Clanwilliam, 8135).

BOTRYTIS CINEREA: DISPERSAL AND INOCULUM

Introduction

Botrytis cinerea [teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel] is a common facultative saprophyte with a wide host range including plants from many different genera and families (Domsch *et al.*, 1980; Holz *et al.*, 2004). Geographically, the pathogen is present throughout the world – wherever suitable hosts are grown (Jarvis, 1977).

Botrytis cinerea is characterised by white mycelial growth producing black, branched conidiophores carrying obovoid, hyaline conidia on swollen conidiogenous cells. Black sclerotia may also be formed, as well as chlamydospores, microconidia and apothecia with asci and ascospores (Domsch *et al.*, 1980; Jarvis, 1980; Holz *et al.*, 2004). All these propagules have the potential to infect susceptible host tissues. The survival, spread and infective ability of different propagules vary between different crops, cropping systems and areas. One such example is the persistence of *B. cinerea* on perennial strawberries (*Fragaria* X *ananassa* Duchesne) through continuous cycling on the leaves (Sutton, 1990). In such a system the conidia and mycelia would be considered very important for pathogen survival. However, with *B. squamosa* Walker on onions (*Allium cepa* L.), sclerotia on organic debris have been shown to play a more important role in pathogen survival (Sutton, 1990). Diverse climatic conditions in different regions can also influence the epidemiological role of different propagules. In general the development of *B. cinerea* is favoured by cool and humid conditions (Wilcox & Seem, 1994). Therefore, in areas with hot, dry summers and cool winters epidemics of *B. cinerea* are often of importance during the winter and the pathogen needs to survive the hot, dry summer.

Propagules and dispersal

The dispersal of inoculum and disease is influenced by several factors. These include the

effects of wind, splashing by rain or irrigation, the activity of insects and the survival of propagules during transport (Gregory, 1968; Kerssies, 1993). Steep dispersal gradients are often observed in close proximity to inoculum sources, but they tend to flatten out with increasing distance from the sources (Gregory, 1968; Aylor, 1987; Chen *et al.*, 2003). This flattening can be ascribed to secondary infections and background contamination. The geometrical form of the source may also play an important role since dispersal gradients around point sources usually tend to be steeper than those around line and area sources (Gregory, 1968). Although the dispersal gradients in the immediate surroundings of larger area sources may be shallow, such sources may exhibit steep dispersal gradients over several kilometres (Fitt *et al.*, 1987). The dispersal gradient downwind from an inoculum source is generally shallower than gradients in the other directions. Despite the fact that most of the inoculum is deposited close to the source, environmental factors can transport a considerable amount of the inoculum over long distances (Fitt *et al.*, 1987).

It is apparent from the preceding discussion that wind and air currents are important factors in the dispersal of diseases caused by *B. cinerea*. This is probably due to the fact that airborne conidia are the most common type of inoculum of *B. cinerea* (Epton & Richmond, 1980). The different types of propagules, however, do differ in their mechanisms of dispersal and also in their infective ability.

Conidia. The hydrophobic conidia of *B. cinerea* are optimally produced at temperatures between 15 and 22°C and high relative humidity (RH) (Jarvis, 1962a, 1977) or continual wetness (Sosa-Alvarez *et al.*, 1995). However, conidia can also be produced at temperatures as low as 5°C, depending on the RH (Sosa-Alvarez *et al.*, 1995). Favourable conditions for sporulation of the fungus is regularly present, so that *Botrytis* is often observed sporulating on senescent and dead tissues in different cropping systems.

Studies on the dispersal of hyphomycete conidia have shown that although fungal conidia are readily dispersed in windy conditions, other environmental factors are also important in the liberation or release of conidia into the air (Chen *et al.*, 2003). The conidia of *B. cinerea* are released from conidiogenous cells when changes in the RH trigger gentle movements of the conidiophores which detach the conidia and allow them to be dispersed by air currents (Jarvis, 1962a). In accordance with this discovery, Hausbeck and Pennypacker (1991) found increases in the concentration of *B. cinerea* conidia in the air among geranium (*Pelargonium* X *hortorum* L.H. Bailey) stock plants when the humidity around the plants was ‘disturbed’. These

disturbances included grower activity around the plants, irrigation with a non-splash, drip system and spraying of fungicides.

In general, conidia of *B. cinerea* are not dispersed over great distances (Chastagner *et al.*, 1978). Conidia produced within a canopy do not easily escape the canopy (Chen *et al.*, 2003; Seyb, 2003; Ahimera *et al.*, 2004). Gusts of wind, however, have the ability to penetrate canopies and release conidia into the atmosphere above the canopy (Aylor, 1990). These conidia may then be dispersed over greater distances by wind.

Although conidia are dispersed mainly through the air by wind and air currents, splashing rain or irrigation and insects have also been found important (Jarvis, 1980; Holz *et al.*, 2004). In hyphomycetes, dispersal gradients up- and down-wind from an inoculum source during fluctuating wind may not differ as much as would be expected (Gregory, 1968). However, increases in wind speed can result in increased liberation of conidia from leaves, although the effect is significantly reduced when the leaves are wet (Chen *et al.*, 2003).

Increases in the number of spores in air are common at the start of rain showers (Hirst & Stedman, 1963). The dispersal of conidia during rain or irrigation still occurs primarily on air currents. The impact of drops on the sporulating material creates shockwaves and air currents that carry the conidia for at least a short distance (Jarvis, 1962a). According to Hirst and Stedman (1963) the increase of spores during rain is only temporary, since continued rain would wash the spores from the air. Consequently the spread of conidia during rain would be very limited. Only a fraction of the conidia are carried on the surface of, or inside drops. Jarvis (1962b) found that drops of water that do carry *B. cinerea* conidia were quite stable and may travel 1 m or more (Jarvis, 1962b). Splash dispersal of several other fungi has also been shown to be very limited (Grove *et al.*, 1985; Chen *et al.*, 2003; Ahimera *et al.*, 2004). Due to biological differences between pathogens, differences in the degree and distances of splash dispersal should be expected (Ahimera *et al.*, 2004).

Several reports of insects vectoring conidia of *Botrytis* spp. have been cited by Jarvis (1977, 1980). Insects have been shown to carry conidia of *B. cinerea* both externally (Fermaud & Gaunt, 1995) as well as in their intestines (Fermaud & Le Menn, 1989; Louis *et al.*, 1996). Conidia inside the insects may be deposited on plant surfaces inside insect faeces or in feeding packages that contain 10 to 50 conidia (Holz *et al.*, 2004).

During dispersal, conidia need to maintain their viability in order to initiate an infection on new hosts. Harrison (1983) reported the loss of infectivity of *B. fabae* Sard. conidia to occur

sooner at higher temperatures (22°C vs. 10°C) and lower RH. The loss of moisture from the spores at low humidity and the depletion of respiratory reagents at higher temperatures were hypothesized to be responsible for decreased infectivity and viability. Ultraviolet and solar radiation has also been found to cause drastic decreases in the viability of *B. cinerea* spores. Aggregates of spores did, however, prove to be significantly more resistant to these environmental factors (Rotem & Aust, 1991). Conidia of *B. fabae* are thought to be able to survive long enough in a light breeze to be dispersed over long distances (Harrison, 1983).

Once deposited on the plant, conidia germinate and infect host plants under conditions of high humidity (Coertze & Holz, 2001) and cool temperatures (Bulger *et al.*, 1987). The ability of *B. cinerea* to sprorulate profusely on diseased material implies that such material could present an important source of secondary inoculum. Hausbeck and Pennypacker (1991), when working on geraniums, reported increases in the incidence of airborne conidia of *B. cinerea* in greenhouses when high numbers of diseased plants were present.

Mycelium. Mycelium of *B. cinerea* inside infected plant material is much more resistant to detrimental environmental influences such as ultraviolet and solar radiation than exposed conidia (Rotem & Aust, 1991). *Botrytis cinerea* has been shown to survive in dead strawberry leaves for several months (Braun & Sutton, 1988). Isolates of *B. cinerea* have also been reported to survive for more than seven months in infected plant debris in fields during summer in Israel (Yunis & Elad, 1989). The nature of the infected material did, however, have a crucial role in the length of the survival period. In rose (*Rosa hybrida* L. cv. 'Mercedes') flowers and tomato (*Lycopersicon esculentum* Mill. cv. 'Faculta 121') fruit, for instance, isolates survived only for 4.5 months. The survival of isolates in artificially inoculated material was also limited (only 9 weeks). In Spain, mycelium appeared to be of great importance in the survival of the pathogen during the summer (Raposo *et al.*, 2001).

Infected, senescent floral parts have been shown to serve as a source of mycelium capable of infection in strawberries (Powelson, 1960). In crops such as grapevine (*Vitis* spp.) where healthy and diseased berries are in close contact, mycelium may also be of major importance as secondary inoculum (Nair & Balasubramaniam, 1995). Mycelium is thought to be more effective as an infective propagule than conidia, since it is not as dependent on free water for infection (Jarvis, 1980).

Sclerotia. Sclerotia are though to be important in the survival of *B. cinerea* (Backhouse & Willetts, 1984; Nair & Martin, 1987). Abundant formation of sclerotia of *B. cinerea* has been observed on grapevine prunings in vineyards in South Africa under wet conditions (Thomas *et al.*, 1981). Such sclerotia have been shown to germinate repeatedly to produce conidia, which serves as primary inoculum in grapevines in Australia (Nair & Nadtotchei, 1987). Sclerotia may also germinate to form apothecia or mycelium (Willetts & Bullock, 1982). Sclerotia of *B. squamosa* in onion fields in Canada usually form late in the season on blighted onion leaves (Sutton, 1990). Although the sclerotia are not directly suitable as infective inoculum, overwintering sclerotia may produce conidia, which are the main source of primary inoculum in diseases such as leaf blight of onion. Sclerotia of *B. squamosa* have been shown to germinate within six days at temperatures between 10 and 20°C to form conidia. The most important limiting factor in such conidiogenic germination is thought to be soil moisture, since sclerotia germinated slower and produced less conidia at low soil moisture levels (Clarkson *et al.*, 2000). The optimal temperature for germination was between 20 and 25°C (Nair & Nadtotchei, 1987).

Braun and Sutton (1987) found very little sclerotia in strawberry fields in Ontario, in contrast to what has been found in Scotland, where many sclerotia that sporulated profusely have been observed (Jarvis, 1962c). They hypothesized that, although sclerotia would present only a minor inoculum source in Ontario, it may become more important when conditions did not favour the survival of mycelium in the leaves. Studies on the survival of sclerotia of *B. cinerea* are controversial. Nair and Nadtotchei (1987) only found 10% survival after 8 months in non-sterile vineyard soil at 22°C and 30% soil moisture in the laboratory, while Raposo *et al.* (2000) found 77% of sclerotia to be viable after 18 months in and around greenhouses.

In areas where *B. cinerea* causes epidemics in winter and needs to survive hot, dry summers, sclerotia are not considered to be of importance in survival, since these structures are not resistant to such high temperatures under dry conditions (Yunis & Elad, 1989).

Ascospores. Apothecia of *B. fuckeliana* have been observed under field conditions in New York bean (*Phaseolus vulgaris* L.) fields (Polach & Abawi, 1975). The presence of apothecia was speculated to indicate the importance of ascospores as primary inoculum in these bean fields. Ascospores are also assumed to be an important source of genetic variation under these conditions. The report mentioned above is, however, the only record of apothecia of *B. fuckeliana* occurring in the field. Consequently ascospores are often disregarded when considering inoculum. Both Jarvis (1980) and Holz *et al.* (2004) have speculated that apothecia

might be an important, but overlooked source of inoculum in the field.

Microconidia. Urbasch (1983a) reported the formation and survival of microconidia of *B. cinerea* under conditions that did not favour the mycelial stage of the pathogen. In some cases appressoria dedifferentiated to form microconidia and consequently lost their ability to infect the host (Urbasch, 1985a). Aggregates of microconidia were sometimes enclosed in a protective coat (Urbasch, 1984). This protective coat appeared to be resistant against chemicals such as alcohol and hydrochloric acid. Therefore, Urbasch (1983a) concluded that these structures might be important in pathogen survival. Infection by germinating microconidia has not yet been reported and although mycelial germination of microconidia has been observed under specific conditions (Urbasch, 1983a), they only formed short germtubes (Urbasch, 1985b).

Chlamydospores. According to Urbasch (1986) chlamydospores could potentially be important to the fungus in several ways, including survival in adverse conditions, dispersal and as infective structures. Germination of these structures could result in the formation of microconidia or mycelium (Urbasch, 1983b).

Park (1954) has shown the development of chlamydospores in certain fungi when the organisms are exposed to adverse conditions that are lethal to fungi that do not produce chlamydospores, implicating these structures in aiding survival. Chlamydospores of *B. cinerea* have been formed in tomato plants under a variety of adverse conditions, but could not survive longer than three months of drought (Urbasch, 1983b).

Primary inoculum

Johnson and Powelson (1983) illustrated the importance of primary inoculum in disease development on snap beans, despite the presence of sufficient amounts of secondary inoculum later in the season. According to Miller and Waggoner (1957) most *B. cinerea* infections in strawberries were caused by inoculum from primary sources nearby. In onions, seed production fields and cull piles have been identified as important sources of primary inoculum (Ellerbrock & Lorbeer, 1977).

In many crops (especially perennial crops), such as grapevines and strawberries, the

primary inoculum of *B. cinerea* is often present within the crop, usually as infected organic debris on the ground (Powell, 1952; Braun & Sutton, 1987; Seyb, 2003). Sporulating colonies of the pathogen have also been observed on senescent leaves of weeds and crop debris on the vineyard floor in kiwifruit [*Actinidia deliciosa* (A. Chev.) C.S. Liang et A.R. Ferguson] (syn. *Actinidia chinensis* Planch) (Michailides & Elmer, 2000) and on wet leaves on the floors of glasshouses (Kerssies *et al.*, 1995). Large-scale production of conidia has also been observed on senescent female cucumber flowers (*Cucumis sativus* L.) (Katan, 1982a). The nature of the organic debris and the conditions in which the material senesced and died, may have an important influence on the sporulation potential of the material (Braun & Sutton, 1988).

In perennial strawberries, quiescent infections have been observed to originate from the infection of asymptomatic leaves. These infections only converted to an aggressive state once the leaves had aged and were starting to die (Braun & Sutton, 1988; Sutton, 1990). Therefore, the incidence of grey mould in annual strawberries can be slightly reduced by removing senescent leaves, which probably serve as an inoculum source (Mertely *et al.*, 2000). Quiescent infections in fruit that are often overlooked during harvest can convert to aggressive infections during storage (Powelson, 1960; Jarvis, 1977).

Besides the inoculum sources mentioned above, seed may also present a potential source of primary inoculum in annual crops such as primula (*Primula* X *polyantha*) (Barnes & Shaw, 2003), chickpea (*Cicer arietinum* L.) (Burgess *et al.*, 1997) and lentil (*Lens culinaris* Medik.) (Morall, 1997). Seedlings produced from infected seed may harbour quiescent infections of the pathogen and only start producing symptoms at a later stage (Barnes & Shaw, 2003).

RESISTANCE OF *B. CINEREA* TO DICARBOXIMIDES

Introduction

Resistance in *B. cinerea* to dicarboximides was reported from several countries in Europe three to four years after this class of fungicides was introduced (Pommer & Lorenz, 1982; Steel & Nair, 1993). Dicarboximide-resistance have since then been reported in *Botrytis* populations from different crops all over the world (Steel & Nair, 1993; Russel, 1995). Cross-resistance has also been observed between different active ingredients of the dicarboximide group (Leroux & Clerjeau, 1985).

The sensitivity of *B. cinerea* to dicarboximide fungicides is encoded by a single major gene called *Daf1* (Faretra & Pollastro, 1991). If the alleles for resistance to dicarboximides were present in a population, the frequency of resistance would represent the interaction between the usage of dicarboximides (selection for resistance alleles) and the degree to which these resistance alleles affect the fitness of the strains possessing them (Beever *et al.*, 1991).

Development of resistance

The development of fungicide-resistance in a pathogen population is dependent on the presence of isolates of the pathogen with natural resistance to the fungicide within the population. Without such initially resistant isolates, resistance to the fungicide in a secluded population (into which no isolates are migrating) would not appear unless resistant isolates develop *de novo* through mutation (Milgroom, 1990). This form of resistance development in *B. cinerea* towards dicarboximides is not believed to occur as readily in the field as in laboratory experiments (Pommer & Lorenz, 1982). However, Oshima *et al.* (2001) suggested that *de novo* mutation has given rise to the evolution of dicarboximide resistance in *B. cinerea* field populations. In fact, another study has shown that at least three independent mutations have occurred in *Daf1* to equip strains of *B. cinerea* with resistance to dicarboximide fungicides (Cui *et al.*, 2004).

Several factors are considered important in the development of resistance. These include the climatic conditions and timing of fungicide applications (Leroux & Clerjeau, 1985). The application of non-related chemicals might also influence the levels of dicarboximide resistance by adding or subtracting to the survival of the dicarboximide-sensitive isolates in relation to resistant isolates (Leroux & Clerjeau, 1985). Incomplete cross-resistance, for instance, between iprodione and folpet have been observed and may result in increases in dicarboximide resistance prior to applications of dicarboximide fungicides (Fourie & Holz, 2001). Beever *et al.* (1991) found that the intensity of disease also had an influence and concluded that the frequency of resistant isolates should remain low in areas with low disease incidence, unless the *B. cinerea* population is exposed to high selection pressure.

When applications of dicarboximides are stopped in grapevines where dicarboximide-resistant *B. cinerea* isolates are present, the frequency of resistant isolates decreases to a minimum of approximately 10% (Löcher *et al.*, 1987; Northover 1988; Pak *et al.*, 1990). Such isolates may persist in the population despite the absence of dicarboximide applications for

several years. When the use of dicarboximide fungicides is resumed in such vineyards, the frequency of resistant isolates has been shown to increase, with greater increases observed with more dicarboximide applications (Pak *et al.*, 1990). In some instances where resistant isolates were present at low levels only one or two applications of dicarboximides were necessary to raise the incidence of resistance to 90–100% (Löcher *et al.*, 1987). These sudden increases in resistance are more likely to occur at the beginning of an epidemic (Skylakakis, 1987).

Stability of resistance

Resistance to dicarboximides in *B. cinerea* has been reported as stable in the absence of dicarboximide fungicides (Katan, 1982a; Vali & Moorman, 1992). According to Katan (1982a), the phenomenon of stable resistance might be explained by large-scale homokaryotization as far as resistance is concerned in resistant isolates. The conversion of a resistant heterokaryotic isolate back to sensitive is hypothesised to occur in the absence of selection pressure, provided that resistant nuclei in the isolate are inferior to sensitive ones, resulting in the loss of resistance. As a result, the majority of resistant isolates in a population where resistance to dicarboximides have previously been reported, would be expected to be of homokaryotic nature, since resistance in heterokaryotic isolates would have been lost during periods where no dicarboximides were applied. In contrast to these findings, Yourman *et al.* (2000) found that the sensitivity levels of *B. cinerea* toward dicarboximides are unstable. They observed changes in resistance levels, both in shifts of sensitive populations toward resistance and vice versa. They also noted higher stability in isolates subcultured on geranium seedlings than those perpetuated on artificial medium.

Fitness and survival of resistant isolates

Fitness and survival of different *B. cinerea* dicarboximide resistant isolates may vary due to the genetically heterogeneous nature of the pathogen (Vali & Moorman, 1992). Accordingly, Pommer and Lorenz (1982) found that the characteristics of resistant isolates are often diverse and may differ considerably. Furthermore, different levels of resistance may also be important when considering the fitness of dicarboximide resistant isolates. Although reports on the fitness and survival of resistant isolates are controversial, indications are that isolates with higher resistance levels exhibit lower fitness levels and *vice versa* (Pommer & Lorenz, 1982;

Panayotakou & Malathrakis, 1983; Fourie & Holz, 1998). Accordingly, strains of *B. cinerea* with high levels of resistance to dicarboximides have not been observed in the field, presumably due to a significant decrease in fitness of these isolates (Pommer & Lorenz, 1982).

Various conflicting reports on the fitness of dicarboximide resistant vs. sensitive isolates exist. Vali and Moorman (1992) found, with *in vitro* tests, similar fitness levels for sensitive and resistant isolates, despite a reduction in virulence and sclerotia-formation by resistant isolates. Similarly, Yunis and Elad (1989) reported that the growth and sporulation of dicarboximide-resistant and -sensitive isolates are similar on a medium selective for *Botrytis*. Research on strawberries has shown that strains of *B. cinerea* with resistance to dicarboximides are able to compete successfully with sensitive isolates as far as the infection of strawberries is concerned, even though resistant isolates exhibit reduced sporulation in comparison to the sensitive isolates (Davis & Dennis, 1981a). Other instances of similar fitness levels have also been reported (Panayotakou & Malathrakis, 1983). Contrarily to these reports, Pommer and Lorenz (1982) found that dicarboximide-resistant isolates often exhibit reduced vitality and pathogenicity when compared to sensitive isolates. Northover (1988) further reported slower growth rates in resistant isolates, whereas similar levels of virulence were found in sensitive and moderately resistant isolates of the pathogen. Beever and Byrde (1982) concluded that although resistant strains seemed ecologically less fit and had a lower virulence than sensitive strains, the levels of fitness and virulence are in some cases similar and may overlap.

Reduced fitness can be manifested in traits such as osmotic sensitivity, slower mycelial growth and reduced sporulation (Davis & Dennis, 1981a, 1981b; Leroux & Clerjeau, 1985; Northover, 1988; Manning & Brook, 1991; Latorre *et al.*, 1994; Fourie & Holz, 1998). Cui *et al.* (2002) found that where mutations in *Daf1* have conferred resistance to dicarboximides, strains of *B. cinerea* have abnormal osmotic sensitivity due to a malfunction in their osmosensing system. This is due to the fact that the *Daf1* gene, which is the target gene for dicarboximide fungicides, is involved in osmoregulation of fungal cells (Cui *et al*., 2002). However, Faretra and Pollastro (1991) reported the differences in osmotic sensitivity between sensitive and resistant isolates to be so small, and that they were in some instances obscured by other phenotypic differences between isolates.

Reports regarding the survival of dicarboximide-resistant *B. cinerea* in relation to sensitive isolates are slightly less diverse. Katan (1982b) reported that dicarboximide-resistant isolates survived through the summer in Israel. Yunis and Elad (1989) have also shown that resistant isolates may survive hot and dry conditions (oversummer) for up to 235 days inside infected debris in the field. Weeds and alternative hosts have also been implied as important in

the survival of resistant strains (Yunis & Elad, 1989). In infected strawberry leaf debris, resistant isolates have also been shown to survive for up to nine months (Davis & Dennis; 1981a). Furthermore, in grapes it has been found that well established dicarboximide resistance can persist through the winter while no dicarboximides are applied (Leroux and Clerjeau, 1985). Observations made on the survival of sclerotia and mycelia of resistant and sensitive isolates of *B. cinerea*, indicate reduced survival in the case of sclerotia of resistant isolates when compared to those from sensitive isolates (Raposo *et al.*, 2000). The survival abilities of mycelia did not differ between the sensitive and resistant isolates. Hsiang and Chastagner (1992), on the other hand, did not find a significant difference between the survival of sclerotia of dicarboximide-sensitive and -resistant isolates and proceeded to prove the ability of sclerotia to maintain resistance through this period.

CONCLUSION

Botrytis cinerea is a serious pathogen that can cause important losses on several crops, including rooibos seedlings. In general, *B. cinerea* prefers cool, humid conditions (Polach & Abawi, 1975). Therefore, *B. cinerea* can be expected to infect and cause disease on rooibos seedlings during the more conducive winter conditions of the Western Cape province. The fungus would need to survive the hot summer months in order to produce primary inoculum at the start of the cooler seasons each year. As the more suitable climate for the proliferation of *B. cinerea* sets in, the production and spread of primary inoculum will become a serious step in the epidemiology, and therefore the control of the disease. Indications are that airborne conidia will be the most important source of inoculum, but mycelia might also be very important (Jarvis, 1980). Another important aspect of the epidemiology of the disease on rooibos seedlings will be the presence of fungicide resistant isolates, and whether they can survive hot summer months in the Western Cape. The survival of *B. cinerea* in such conditions have been recorded both for isolates sensitive (Raposo *et al.*, 2001) and resistant toward dicarboximides (Yunis & Elad, 1989).

Considering what is known in several crop systems about the epidemiology of *B. cinerea*, it should be possible to improve cultural management of *B. cinerea* diseases by removing primary inoculum sources. In order to do this, these sources need to be identified with the aid of epidemiological information pertaining to *B. cinerea* in the specific cropping system. However, no research has been done on the epidemiology of *B. cinerea* in rooibos nurseries. A study

should therefore be conducted to investigate the inoculum ecology of *B. cinerea* in rooibos nurseries in order to identify primary sources of inoculum and to improve the environmentally friendly management of the disease. Since resistance of the pathogen toward dicarboximides has been found to be stable, this trait may be utilised as an aid in tracking the movement of the fungus.

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Figure 1. A rooibos nursery

Figure 2. Grey mould symptoms on rooibos seedlings

Figure 3. Sporulation of *B. cinerea* on rooibos seedlings affected by grey mould

2. THE INOCULUM ECOLOGY OF *BOTRYTIS CINEREA* IN ROOIBOS NURSERIES

ABSTRACT

Grey mould (*Botrytis cinerea*), the most important foliar disease of rooibos seedlings, is primarily controlled with fungicides. The aim of this study was to investigate the ecology of the pathogen in rooibos nurseries in order to improve environmentally friendly management of the disease. The study was conducted in four nurseries over two production seasons (March to July 2003 and 2004). Levels of airborne inoculum of *B. cinerea* were monitored on a monthly basis inside and around the nurseries with spore traps. Samples of plant material and organic debris were taken in the corresponding areas to determine the incidence of plant material infected by the pathogen and the incidences of grey mould in the nurseries were recorded. Relatively low numbers of *B. cinerea* colonies were observed on the spore traps. Similar levels of airborne inoculum were observed inside and around the nurseries. The incidence of plant material yielding *B. cinerea* was consistently higher outside the nurseries than inside, indicating the importance of such material as potential sources of inoculum. Since patterns of airborne inoculum observed in this study confirmed reports of the local dispersal of *B. cinerea*, the removal of possible hosts outside the nurseries could aid in the management of grey mould in rooibos nurseries.

INTRODUCTION

In the Western Cape province of South Africa, grey mould, caused by *Botrytis cinerea* Pers. ex Pers., is the most important foliar disease of rooibos [*Aspalathus linearis* (Burm. F.) R. Dahlg.] seedlings. The fungus typically attacks the lower stems and leaves of older seedlings (2 months and older) causing wilt-like symptoms and death. Disease occurrence during this part of the season (May to July) coincides with the moderate, Mediterranean climate of Western Cape winters. The onset of disease is furthermore encouraged by the denser canopy formed by older seedlings. This canopy not only creates a suitable microclimate for the development of *B. cinerea* (higher humidity, lessened penetration by fungicides, etc.), but also possibly predisposes the lower plant parts to infection due to reduced light intensities (Zhang *et al.*, 1995; Hausbeck & Moorman, 1996).

Botrytis cinerea can produce three kinds of inoculum: conidia, ascospores and mycelia (Jarvis, 1980). Since ascospores are rarely observed in the field (Polach & Abawi, 1975), conidia and mycelia are considered to be the most important forms of inoculum (Jarvis, 1980). Mycelia are less dependent on free water for survival and penetration and are therefore considered to be more effective than conidia in causing disease (Sirjusingh *et al.*, 1996). Conidia, on the other hand, are by far the most common form of inoculum (Epton & Richmond, 1980; Hausbeck & Moorman, 1996). The conidia are mainly dispersed in air currents and on or in drops of water (Jarvis, 1980). In some cropping systems vectoring of *B. cinerea* by insects may also be an important mechanism of dispersal (Louis *et al*., 1996). In grape vineyards, it has been shown that conidia from ground sources had steep deposition gradients on the ground, while once in the air, it could be distributed over long distances (Seyb, 2003). Since conidia of *B. cinerea* are mainly dispersed locally (Chastagner *et al.*, 1978), it should be possible to minimise the incidence of grey mould through effective management of inoculum sources. However, the main inoculum sources and the inoculum dispersal of *B. cinerea* would be expected to differ between different crops and geographical sites, due to differences in the environment and surrounding vegetation of different agricultural systems and even different farms.

Once deposited on the host, the conidia can germinate and infect the plant under favourable conditions e.g. cool temperatures and high humidity (Jarvis, 1977). Latent infections of *B. cinerea* have been reported on several crops. Such infections may maintain an asymptomatic presence in the host until a certain phenological stage is reached before symptoms are observed (Verhoeff, 1980). In primula (*Primula* X *polyantha*) it has been shown that latent infections originating from infected seed may persist in seedlings without producing symptoms until the plants reach flowering age (Barnes & Shaw, 2002). Physiological changes in infected tissues, such as desiccation or other forms of stress, might also trigger the development of symptoms (Jarvis, 1977). Lamprecht and Denman (1995), however, found that *B. cinerea* is not present in seeds of rooibos.

Botrytis cinerea is not a host-specific organism and has been found on numerous crops, including several weeds and wild plant species (Crous *et al.*, 2000). Such alternative hosts may well serve as sources of inoculum. Senescent plant material often serves as an ideal substrate for the development and sporulation of *B. cinerea* (Michailides & Elmer, 2000). Sporulating colonies of the fungus have also been observed on organic debris in rooibos nurseries (S.C. Lamprecht, ARC–PPRI, Private Bag X5017, Stellenbosch, 7599, personal communication).

The aims of this study were to determine in four rooibos nurseries at monthly intervals

(March to July) over two seasons (2003, 2004) (i) the number of diseased rooibos seedling patches inside nurseries, (ii) the incidence of viable *B. cinerea* in the air in and around nurseries, and (iii) the incidence of *B. cinerea* on plant material collected in and around nurseries. The data was used to identify possible inoculum sources in rooibos nurseries.

MATERIALS AND METHODS

Nurseries. The survey was conducted in four rooibos nurseries in the Clanwilliam-area. Although all four nurseries have a Mediterranean type climate, two of the nurseries (nurseries A and D) were situated in a higher rainfall area (ca. 450 mm per year). Rooibos seeds were sown in the nurseries from late February to middle March each year. The seeds sown in all the nurseries were scarred mechanically to improve germination. The layout of each nursery complied with recommendations given to seedling producers by Rooibos Ltd. (P.O. Box 64, Clanwilliam, 8135) i.e. an east-west row orientation, 10 cm spacing between rows and a sowing density of approximately 15 g/m. Sprinkler irrigation systems were used in all the nurseries on a daily basis. Seedlings were fertilized with limestone ammoniumnitrate (28% N, WPK Agriculture Ltd., Cape Town, South Africa) six and ten weeks after sowing in nurseries A, B and C (during 2003) and with an organic formulation of chicken manure (Neutrog Africa, Stellenbosch, South Africa) in nurseries C (during 2004) and D. A high level of sanitation was maintained in the four nurseries. Spray programmes recommended by Rooibos Ltd. were followed in all nurseries (Table 1). The programme followed in nurseries B, C and D involve weekly sprays with fungicides such as chlorothalonil (Bravo 50 SC, Effekto) and captab (Captab 50 SC, Dow AgroSciences) from 1 to 10 weeks after sowing. From 11 weeks after sowing pyrimethanil (Scala 40 SC, Bayer) and a combination of iprodione (Rovral Flo 25 SC, Bayer) and chlorothalonil were applied in alternating weeks until the seedlings were transplanted. An alternative to this programme (followed in nursery A) replaced pyrimethanil applications with iprodione (Table 1).

Sampling strategy. A crude map of each nursery was drawn up and the area covered by the nursery was divided into 50 sampling locations of more or less equal size. The surrounding area up to 20 m from the border of the nursery was also divided into 50 even-sized sampling locations. A code was allocated to each sampling location to indicate its exact position. This code contained a letter indicating the nursery, a letter to indicate whether the position is inside (I)

or outside (O) the nursery and a number allocated to a specific sampling location on the map of the nursery. The amount of viable *B. cinerea* in the air and on plant material was estimated and the number of diseased patches recorded at each sampling location at monthly intervals (March to July) over two seasons (2003, 2004). Sampling was usually conducted within the last two weeks of each month.

Sampling *B. cinerea* from air. Spore traps for sampling *B. cinerea* from air, consisted of small Petri dishes (65 mm diameter) filled with Kerssies' *B. cinerea* selective medium (Kerssies, 1990). The spore traps were prepared within a week before use and kept at approximately 15°C until exposed in the nurseries. The bottom half of each dish was marked with the date of exposure as well as the code corresponding to the sampling location where it would be placed. Each small Petri dish was placed inside the empty bottom half of a large Petri dish (90 mm diameter) that was fastened to an iron rod. The large Petri dish was fastened at an angle to the top of each iron rod with the aid of a rubber cork and galvanized wire (Figure 1). The slant of the dish prevented the accumulation of rainwater in spore traps while still allowing for sedimentation of inoculum. A piece of builder's line was fastened across the breadth of the large Petri dish to prevent spore traps from falling out. The rods were placed at their specific locations at the beginning of each season and left there for the duration of the season. Rods placed outside the nurseries were impaled in the ground to such an extent that the large Petri dishes were approximately 1 m above the ground while the dishes inside the nursery were kept just above seedling canopies. Since the movement of tractors in the nursery and livestock around the nursery occasionally caused damage to the dishes, it was necessary to check all the rods at the beginning of each sampling period. Any broken dishes would then be replaced by new ones, the positions of any misplaced rods would be corrected and the height of the dishes inside the nursery was adjusted according to seedling size.

Spore traps were placed in the nurseries on each of the first three days of every sampling period. Starting at approximately 13:30 in Nursery B, the bottom halves of the spore traps were placed in the larger Petri dishes at their specific locations. Nurseries D, C and A followed at approximately 14:50, 15:15 and 16:00 respectively. In some cases placement of spore traps coincided with nursery irrigations. Therefore, to prevent unnecessary exposure of the selective medium to water, the spore traps would then be placed out without removing the lids and one of the farm labourers would remove the lids after irrigation. Dishes were collected the following morning by either the farmer or another member of the farm personnel, with specific instructions not to touch the medium and to replace the lid of each spore trap at the location of the

corresponding rod. This was done between 07:30 and 09:00, before seedling irrigation. The spore traps were then placed in a cardboard box and left in a cool area until collection. Once collected, the dishes were placed in insulated containers and cooled with frozen ice packs.

On the evening of the fourth day, the spore traps were unpacked and incubated at 22°C. Due to the dry climate of the Cedarberg Mountains, some of the spore traps had dried out considerably during exposure in the nurseries. It was attempted to rehydrate these dishes with a few millilitres of sterile, de-ionised water, but since this treatment had little or no effect on the amount of fungal growth developing on the dishes, such spore traps were discarded without evaluation. After a period of 11–13 days, each dish was examined with the aid of a dissecting microscope and the number of sporulating colonies of *B. cinerea* was recorded for each dish. Spore traps containing non-sporulating colonies resembling *B. cinerea* were set aside and re-evaluated (only once) the following day. The mean of the number of colonies observed across the three days of each sampling period was calculated for each sampling location.

Assessment of diseased rooibos seedling patches within nurseries. During the first two days of sampling, as the spore traps were being placed out, nurseries were inspected for the presence of seedlings affected by grey mould. Such diseased seedlings were identified by wilt-like symptoms combined with the presence of fungal growth resembling *B. cinerea* on the lower plant parts. A diseased patch consisted of any number of adjacent seedlings displaying symptoms and signs characteristic of grey mould, sometimes including seedlings that had already died. The nurseries in which diseased seedlings were found, were systematically inspected on the third day and the number of diseased patches at each sampling location was recorded.

Plant material sampling in and outside nurseries. Aside from diseased rooibos seedlings, material from other plant species was collected on the fourth day of sampling in and around each nursery to determine inoculum sources in addition to diseased rooibos seedlings. Plant material collected inside nurseries consisted of two asymptomatic seedlings, four pieces of organic debris and four weeds collected at each sampling location per nursery. During 2004 additional samples were taken of two asymptomatic cotyledons (both from the same seedling) and two senescent cotyledons from the nursery bed. Random sampling within each sampling location was encouraged. All plant material was placed in insulated containers with three or four frozen ice packs to maintain a low temperature during transport. On the evening of the fourth

day, the containers with the plant material was opened and stored at 4°C.

Plant material collected outside nurseries included several agricultural crops as well as natural vegetation (weeds, trees and shrubs) and will collectively be referred to as vegetation from here on. Outside the nursery the sampling locations were grouped together as to indicate different groups of vegetation. Samples of the most common plant species within each group was taken and placed in a large plastic bag labelled with the codes of the corresponding sampling locations. It was attempted to keep the number of samples of each plant species at each group of sampling locations relatively constant, but since the incidence of certain weeds differed as the season progressed, this was not always possible. Plant material was stored similar to material collected inside the nurseries.

Processing and incubation of plant material for induction of *B. cinerea* sporulation. Asymptomatic seedlings were unpacked and treated on the first day after sampling. The roots of the seedlings were removed 1–2 cm below the crown. One seedling (labelled 'Sterile') from each sampling location was surface-sterilised (30 s in 70% ethanol, 2 min. in 0.35% sodium hypochlorite, 30 s in 70 % ethanol) and air-dried. The other seedling was labelled 'Non-sterile' and was left untreated. Both groups of seedlings were immersed in paraquat solution (WPK Paraquat, 200g/l [bipyridyl], WPK Agricultural, Cape Town, South Africa) for 30 seconds, rinsed in de-ionised water and air-dried. This was done to suppress host-resistance and encourage the development of fungi on plant material (Grindrat & Pezet, 1994).

Weeds collected in and outside nurseries, vegetation collected outside nurseries and cotyledons collected from live rooibos seedlings were unpacked and processed respectively on the fourth, fifth and sixth days after sampling. All these samples were surface-sterilised and treated with paraquat as described above.

Senescent cotyledons, organic debris and diseased seedlings were unpacked on the seventh day after sampling in a similar way as the living samples, but were not sterilised or paraquat treated. Sterile tweezers were used when unpacking the diseased seedlings to prevent cross-contamination of isolates between different diseased seedlings.

All plant material samples were placed on epoxy-coated steel mesh screens (53 x 28 x 3 cm) that had been covered by paper towels. The samples were arranged in such a way as not to make contact with each other. A block was drawn around each plant sample on the paper towels covering the mesh screens and the code of the appropriate sampling location was written down in

the block.

The mesh screens containing the various samples were placed in ethanol-disinfected perspex (Cape Plastics, Cape Town, South Africa) chambers (31 x 62 x 56 cm) lined with a sheet of chromatography paper with the base resting in de-ionised water to establish high relative humidity (≥ 93% RH). The material was incubated in the moist chambers at 22°C for 14 days. Moist chambers containing the organic debris and cotyledons (both living and senescent) were atomised daily with de-ionised water to further increase the RH in these chambers. The plant material was inspected daily and any other material showing signs of desiccation was also atomised with de-ionised water.

Following incubation, the plant material was inspected with a dissecting microscope. The presence or absence of sporulating *B. cinerea* was recorded on the sterile and non-sterile asymptomatic seedlings as well as on the living and senescent cotyledons. The incidence of sporulating *B. cinerea* on each of these samples as well as on the other plant material (with the exception of the diseased seedlings) was recorded as the number of affected samples in relation to the number of samples taken at each sampling location. The incidence of *B. cinerea* on vegetation and weeds outside the nurseries was calculated similarly for each sampling location or group of sampling locations. The data collected from all the plant material at each sampling location were combined into one value representing the percentage incidence of *B. cinerea* on the plant material at that sampling location.

Statistical analysis. The survey consisted of a 2 × 4 × 5 × 2 factorial with two seasons (2003, 2004), four nurseries (A, B, C, D), five months (March – July) and two positions (inside, outside). The number of diseased patches (NDP) was log transformed [LNDP = $\log_{10}$(NDP + 1)] and the incidence of *B. cinerea* on plant material was transformed to percentage and logits. The mean number of colonies on spore traps did not undergo any transformation. These variables were subjected to a factorial analysis of variance using SAS V8.2 (SAS, 1999). Shapiro-Wilk's test was performed to test for non-normality (Shapiro & Wilk, 1965). The Student's t–LSD was calculated at a 5% significance level to compare means of significant interactions or main effects. Pearson correlations between the variables were also calculated (Cochran & Cox, 1957).

It is likely that inoculum observed under field conditions originates only in part from traceable, local sources and many distant sources may have contributed to the total amount (Gregory, 1968). To lessen the effect of such "background contamination", the statistical analysis was repeated with data only from sampling locations where contaminated plant material

(symptomatic or asymptomatic seedlings, weeds, organic debris, dead or living cotyledons or natural vegetation) was found. To distinguish between the two analyses, they will henceforth be referred to as 'analysis A' (including all data) and 'analysis B' (excluding zeros). The exclusion of data in analysis B resulted in an unreliable representation of the incidence of *B. cinerea* on plant material in and around the nurseries. Consequently analysis B will only be included in the results and discussion of data collected on the spore traps.

Deviations from normality in either analysis was found to be due to kurtosis and the inclusion of many zeros in the analysis and the results was therefore deemed suitable for interpretation (Glass *et al.*, 1972).

RESULTS

Sampling *B. cinerea* from air. Colonies of *B. cinerea* developed on spore traps from all nurseries and all sampling periods. The number of colonies varied greatly between different years, months, days, nurseries and also within nurseries. No clear patterns could be distinguished in the incidence of airborne *Botrytis* when considering the separate sampling locations.

The analyses of variance (ANOVAs) for the influence of year, nursery, month and position on the incidence of *B. cinerea* on the spore traps over the three consecutive days indicated significant interactions between the different years, nurseries and months in both analyses ($P < 0.0001$ for both analyses) (Table 2). Significant interactions were also observed between the months and positions (analysis A: $P = 0.0075$; analysis B: $P = 0.0009$). In general, higher incidences of the pathogen on the spore traps were found during April and May with lower levels occurring in March, June and July. In analysis A the interaction between the nurseries and positions was also significant ($P = 0.0031$).

Analysis A (including all data) revealed no significant differences ($P = 0.05$) between the amounts of inoculum recorded inside and outside the nurseries, although a slight change was observed towards the end of the season: the inoculum dosages outside the nurseries increased slightly, whereas the dosages inside decreased (Table 3). Such slight differences became more pronounced in analysis B (excluding zeros). The pattern of the incidence of airborne inoculum outside the nurseries did not undergo any changes, but the data inside the nurseries showed some interesting changes. Instead of a peak in April and a slight decrease toward May as was

observed during analysis A, a more gradual increase in airborne inoculum was observed toward a slight peak in May (reaching a mean value of 1.30 cfu's per spore trap per day), after which a gradual decline followed toward July (Table 4). These changes resulted in a significant difference ($P = 0.05$) between the means recorded inside and outside the nurseries in June.

Accessing the number of diseased rooibos seedling patches within nurseries. In each year diseased patches were only observed in two nurseries (nurseries B and C during 2003 and nurseries A and C during 2004) and only in the later part of the season. Very few diseased patches were observed in 2003. The results obtained with the transformed data were similar to those obtained with the original data, therefore only the transformed data (LNDP) will be discussed.

The analysis of variance for the variables year, nursery and month revealed significant interactions ($P < 0.0001$) between all the influences involved (year, nursery and month) regardless of the variable used (Table 5). The incidence of grey mould in nursery C (but not in nursery B) was significantly higher ($P = 0.05$) than in the other nurseries during 2003 (Table 6). In 2004 diseased seedlings were already noted in May in nursery C. The high numbers of diseased patches in nurseries A and C during 2004 were significant when compared to nurseries B and D, and significantly different when compared to each other.

Highly significant, but slight negative correlations were observed between LNDP and the means recorded on the spore traps in analysis A ($r = -0.095$, $P < 0.0001$, $n = 1742$) and B ($r = -0.29202$, $P = < 0.0001$, $n = 337$). The correlation between LNDP and the percentage infection on the plant material (only analysis A) was, although very slight, positive and significant ($r = 0.076$, $P = 0.0014$, $n = 1744$).

Incidence of *B. cinerea* on plant material (excluding diseased rooibos seedlings). The results of the statistical analysis with the transformation of the incidence of *B. cinerea* on the plant material (asymptomatic rooibos seedlings, organic debris, weeds, rooibos cotyledons and vegetation) to the percentage and logits did not differ from that observed when the original percentage values were used. Only the analysis with the percentage values will therefore be discussed.

The analysis of variance (ANOVA) for the effects of year, nursery, month and position

concerning the incidence of *B. cinerea* on plant material (Table 7) indicated significant ($P < 0.0001$) interactions between all four variables (year, nursery, month and position). During 2003 the incidences of the pathogen on plant material outside the nurseries were significantly ($P = 0.05$) higher on plant material collected inside the nurseries during all months with the exception of nursery A in July, where a sharp drop in the incidence of *B. cinerea* outside the nursery occurred (Table 8). None of the values observed inside the four nurseries during 2003 differed significantly. During 2004 the situation was similar, but significantly higher values were observed inside nursery A during May and June than were observed during the rest of the year in any of the nurseries (Table 8). Significant monthly differences in the positional incidence of *B. cinerea* were observed during 2004 in all nurseries except for nurseries A, B and D in March, nursery A in May and nursery B in June.

Significant, but slight positive correlations were observed between the mean incidence of *B. cinerea* on the spore traps and the incidence of the pathogen on plant material both in analysis A ($r = 0.054$, $P = 0.0178$, $n = 1891$) and B ($r = 0.109$, $P = 0.0158$, $n = 486$).

DISCUSSION

Conidia of *B. cinerea* observed above crop canopies generally reach a maximum during late morning or early afternoon when evaporating dew lower the humidity around conidiophores to trigger the release of conidia (Jarvis, 1962). During this study, however, the distance between the nurseries (i.e. the time needed to travel between nurseries) and the timing of irrigations in the nurseries prevented the sampling of airborne inoculum during such peak-times. The data observed on the spore traps should therefore not be considered as the maximum potential inoculum dosages.

The significant three-factor interaction (year × nursery × month) in the analyses of the airborne incidence of *B. cinerea* is believed to be caused by uncharacteristically high incidences of *B. cinerea* on the spore traps in 2004 in nursery B during May and in nursery A during June (data not shown). The location of nursery B (low rainfall) in a completely different area from that of the other three nurseries (high rainfall) could have been the cause of the high mean observed in May 2004 in this nursery. The high value observed in nursery A during June 2004 could be attributed to a high incidence of grey mould in this nursery during the latter part of the 2004 season.

The two-factor interaction involving the airborne isolates observed in each of the nurseries at the different positions was only significant in analysis A. The significance of this interaction could be due to differences between the amount of airborne inoculum in- and outside nurseries A and C (the two nurseries where a high incidence of grey mould was observed) (data not shown). In nursery A the incidence of airborne *B. cinerea* inside the nursery was higher than outside and could have been caused by secondary inoculum produced on diseased seedlings in the nursery. Although a relatively high incidence of grey mould was also observed in nursery C, the incidence of airborne *B. cinerea* outside the nursery was higher than that observed inside. This might indicate the presence sources of conidia outside the nursery even though no sporulating material was observed outside the nursery. The higher levels outside the nursery is therefore strange, since the verified production of conidia on diseased seedlings inside the nursery would be expected to raise the airborne inoculum inside the nursery above the levels recorded outside.

Several studies have shown that conidia of *B. cinerea* are only dispersed locally (Johnson & Powelson, 1983; Seyb 2003). Consequently one would expect the values of airborne inoculum within the nurseries to differ from those outside, since the availability of inoculum sources would undoubtedly differ due to sanitation practices inside the nurseries. The fact that such a difference was not observed in analysis A indicates therefore that the majority of inoculum recorded on the spore traps is so-called "background contamination", i.e. inoculum from non-specific sources which are not located in the immediate surroundings of the area sampled (Gregory, 1968). This assumption is reinforced by the fact that the pattern observed inside the nurseries changed when the data was analysed without the zeros (analysis B). The fact that the patterns observed outside the nurseries did not change, could indicate that the spore traps outside the nurseries were too high above the ground to capture inoculum from important ground-based sources outside the nurseries. Alternatively, the sources outside the nurseries could be sufficiently representative of the 'natural' sources of the background contamination to merit the similarity.

The change in the pattern of the airborne inoculum inside the nurseries when analysed without the zeros (analysis B) shows that there are at least some inoculum sources within the nurseries. The only observed material that sustained sporulating *B. cinerea* in the field during this study was diseased seedlings, but *Botrytis* sporulation have been observed on organic debris in rooibos nurseries previously (S.C. Lamprecht, personal communication). Seedlings affected by grey mould are an obvious source of secondary inoculum as can be seen by the significantly higher levels of airborne inoculum inside the nurseries in comparison to that observed outside in

June – the first month in each season where high levels of diseased seedlings were observed. Cultivation practices, such as sprinkler irrigation and spraying of fungicides, associated with the production of rooibos seedlings could encourage the spread of such secondary inoculum by contributing to changes in the humidity (a factor involved in releasing conidia from the conidiophores) and turbulence in the seedling canopy (Hausbeck & Pennypacker, 1991). In some instances, the weight of water clinging to the foliage of the seedlings drags the seedlings toward the soil, causing a temporary kind of 'damping-off' which results in the canopy opening up and sporulating tissue near the ground being exposed. This would further encourage dispersal of the conidia.

Correlations of the incidence of disease with the amount of airborne inoculum, were, however, negative in both analyses. These statistics seem illogical and were probably influenced by the low incidence of disease in 2003 as well as a severe drought in the area during both seasons, which resulted in the inclusion of many zeros in the analysis. Grey mould in the nurseries could easily have spread through mycelium, since the density of seedlings in the nurseries are quite high and dispersal through direct contact is very likely. The quantification of disease by counting the diseased 'patches' instead of individual diseased plants would have compensated for this mechanism of spread and it is therefore thought that mycelial spread did not influence the results significantly. Disease dispersal with the aid of insects is also thought to be of little significance, since insect pests are of little importance in rooibos nurseries.

A possible explanation for the negative correlations might be found in the high incidence of airborne inoculum in April and May. According to Lamprecht (1996) grey mould is usually only a problem in nurseries from May to July, when seedlings reach an age of 2–4 months during (an observation confirmed in this study; see Table 6). The reason for this has not been thoroughly investigated, but it is probably related to changes in the climatic conditions during the year. The cool, humid conditions preferred by *B. cinerea* for infection (Blakeman, 1980), is more common in the winter (June and July) in the Western Cape, while March, April and May are dryer and hotter. Besides the macroclimate, the smaller size of the seedlings during March, April and May result in a less dense canopy and therefore lower humidity around the lower plant parts, which are typically affected by grey mould. Since susceptibility of several crops to *Botrytis* have been shown to be linked to the physiological age of the plant or plant organ (Jarvis, 1980; Verhoeff, 1980; Braun & Sutton, 1988; Hausbeck & Moorman, 1996), it is also possible that the younger seedlings are more resistant to infection by *B. cinerea*, although no studies have been done to confirm or reject this possibility. It can therefore be said that the three components necessary for successful symptom expression (climate, host and pathogen) (Agrios, 1997) might

not have interacted favourably for the development of grey mould during April and May even though high levels of inoculum were present in the air.

When considering the percentage of infected plant material, two important facts should be taken into account. Firstly, the samples of plant material (with the exception of organic debris) were taken from asymptomatic plants and would therefore only represent potential inoculum sources which may only function as active sources when the infected material start expressing symptoms or have died. Secondly, as was mentioned earlier, the incidence of *B. cinerea* on plant material in the nurseries as presented by analysis B should not be seen as a reliable rendition of the actual level of infection in the nurseries since most of the material on which the pathogen was not found were not included in the calculations. The discussion of the incidence of *Botrytis* on the plant material will therefore focus mainly on results obtained with analysis A.

It is of note that, with the exception of nursery A in 2004, the incidences of *B. cinerea* on plant material outside the nurseries were constantly higher than that observed inside the nurseries. Daily irrigations in the nurseries should create an environment more suitable to infection and sporulation of the pathogen than that of the dry, hot environment outside the nurseries. Frequent applications of captab were made in all nurseries during the first part of each season. Although this fungicide is not applied as a botryticide in rooibos nurseries, it has been shown to suppress the development of *B. cinerea* and grey mould in other crops (Smith, 1998; Haydu & Legard, 2003) and could have done the same in rooibos nurseries. Consequently it could be said that the low incidence of *B. cinerea* inside the nurseries would indicate very few potential inoculum sources within the nurseries.

The erratic pattern in the incidence of *B. cinerea* on plant material outside the nurseries could probably be explained by the fact that the nature of the samples (different plant species and the number of each sampled) differed considerably between the nurseries and in some cases also between consecutive months for the same nursery. It is likely that the availability and susceptibility of specific alternative hosts for *B. cinerea* would differ from nursery to nursery and from month to month. Consequently the data concerning the plant material collected outside the nurseries suffice mainly as an indication of the presence of alternative hosts, and therefore potential inoculum sources, outside the nurseries. The combined effect of the percentage infected plant material in- and outside the nurseries showed a significant positive correlation to LNDP, suggesting that either the infected plant material (asymptomatic rooibos seedlings, organic debris, weeds, rooibos cotyledons & vegetation) served as an inoculum source for infection of the seedlings, or the diseased rooibos seedlings served as a source of inoculum for

infection of other plant material. To distinguish between the two possibilities the situation in nursery A in 2004 will be used as an example.

In this nursery sanitation practices as far as weed control inside the nursery is concerned, was neglected in May 2004 resulting in the presence of many large weeds inside the nursery during sampling in May. The coinciding increase in infected plant material observed inside the nursery for this month is very prominent, and did not differ significantly from the corresponding level of infected material outside the nursery. A large part of the infected material inside the nursery was comprised of two weeds, namely wild radish (*Raphanus raphanistrum* L.) and devil's thorn (*Emex australis* Steinh.). *Botrytis cinerea* was also often observed on these two species outside the nursery. A large, dense stand of wild radish was present next to nursery A and devil's thorn was also frequently observed in close proximity to the nursery. A further interesting observation was the incidence of grey mould in this nursery in the following month (June). The presence of *B. cinerea* on the plant material before grey mould was noticed in the nursery indicates the importance of infected plant material inside or close to the nursery as a source of primary inoculum. The movement of primary inoculum is, however, still unclear. Three possibilities will be discussed.

Firstly, if *B. cinerea* is seed-borne in especially the two weeds mentioned above, it is possible that infected seeds present in the nursery soil from the beginning of the season may germinate and result in the presence of infected weeds inside the nursery which may then serve as the source of primary inoculum if weed control is not diligently practised. Secondly, (also if *B. cinerea* is seed-borne in the weeds involved) the presence of infected weeds both in- and outside the nursery could indicate that infected seeds produced outside the nursery spread into the nursery to establish infected weeds which may serve as primary inoculum sources. Seed-borne infections of *B. cinerea* have been reported for crops such as lentil (*Lens culinaris* Medik.) (Morall, 1997) and primula (Barnes & Shaw, 2003). Tests on the seed-borne aspect of *B. cinerea* in the two weeds under discussion are, however, necessary to confirm or reject these hypotheses. The survival and dispersal of *B. cinerea* through infected rooibos seed is not thought to be of importance (Lamprecht & Denman, 1995). Thirdly, it is possible that dense stands of infected weeds could serve directly as primary inoculum sources and *B. cinerea* is dispersed into the nursery as conidia or infected debris originating from the weeds. Literature on the dispersal of *Botrytis* conidia suggests that a very small amount of conidia escapes canopies (such as the dense canopy that would be formed by a thick stand of wild radish) (Chastagner *et al.*, 1978) and that the dispersal of conidia from a ground source (such as the low-growing devil's thorn) is also very limited (Jarvis, 1962; Seyb, 2003). Consequently, the dispersal of *B.*

cinerea as infected debris may be important in the development of grey mould in rooibos nurseries. Organic debris sampled inside the nurseries during this survey generally harboured little or no *B. cinerea*. The nature of the sampled material was for the most part at least as large as a small leaf. If the third possibility is of importance, the organic debris vectoring the pathogen would have to be considerably smaller than the sampled debris, possibly parts of flowers, small leaf fragments or maybe even pollen. Such small fragments would probably be more suitable for dispersal under natural conditions. The data collected during this study, is not, however, sufficient to determine which of the three possibilities would be applicable in the nursery situation.

In each of the three scenarios mentioned above, sanitation is an important mechanism by which disease could be suppressed. This is further suggested by the fact that nurseries that had the highest number of diseased rooibos seedlings in June also had the highest incidence of *B. cinerea* on plant material (asymptomatic rooibos seedlings, organic debris, weeds, rooibos cotyledons & vegetation) in- and outside the nursery in May and June. The development of grey mould in nursery A has illustrated the importance of sanitation inside the nurseries, but if the second or third possibilities are true, sanitation outside the nurseries might also have an important impact. Potential suppression of *B. cinerea* in the soil by soil solarisation is an environmentally friendly option that might be worth looking into. Not only should this management strategy kill sclerotia and mycelium of *B. cinerea* in the soil (López-Herrera *et al.*, 1994) but the viability of seeds of weeds in the soil would also decrease significantly. Including an area stretching approximately 10 m from the border of the area intended for a rooibos nursery in the solarisation might therefore not only decrease the amount of primary inoculum in the immediate surrounds of the nursery, but also aid in weed control both in- and outside the nursery.

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Table 1. Program for the application of fungicides in rooibos nurseries as recommended by Rooibos Ltd.

Application (days after sowing)	**Fungicides**[y]
7 (1 week)	captab
10 days	captab (drench)
14 (2 weeks)	captab
21 (3 weeks)	captab
24 days	captab (drench)
28 (4 weeks)	captab & iprodione
35 (5 weeks)	captab
42 (6 weeks)	captab & chlorothalonil
49 (7 weeks)	captab & chlorothalonil
56 (8 weeks)	captab & chlorothalonil
63 (9 weeks)	captab & chlorothalonil
70 (10 weeks)	captab & chlorothalonil
77 (11 weeks)	pyrimethanil (or iprodione)[z]
84 (12 weeks)	chlorothalonil & iprodione
91 (13 weeks)	pyrimethanil (or iprodione)
98 (14 weeks)	chlorothalonil & iprodione
105 (15 weeks)	pyrimethanil (or iprodione)
112 (16 weeks)	chlorothalonil & iprodione

[y] captab = Captab (Dow Agrosciences) at 0.5g a.i./0.5l/m^2; captab (drench) at 2g a.i./2l/m^2; chlorothalonil = Bravo (Effekto) at 0.5ml a.i./0.5l/m2; iprodione = Rovral Flo (Bayer) at 0.25ml a.i./0.5l/m^2; pyrimethanil = Scala (Bayer) at 0.4 g a.i./0.5l/m^2

[z] Pyrimethanil applications were substituted for iprodione applications in nursery A.

Table 2. Analysis of variance on the mean number of *Botrytis cinerea* colonies on spore traps in and around rooibos nurseries from April to July in 2003 and from March to July in 2004

	Analysis					
	A[y]			B[z]		
Source of Variation	**df**	**MS**	**P**	**df**	**MS**	**P**
Year (Y)	1	15.715	<0.0001	1	0.006	0.8874
Nursery (N)	3	10.996	<0.0001	3	3.713	<0.0001
Y × N	3	5.213	<0.0001	3	5.516	<0.0001
Month (M)	4	109.977	<0.0001	4	15.705	<0.0001
Y × M	3	65.134	<0.0001	3	17.486	<0.0001
N × M	12	8.643	<0.0001	12	4.590	<0.0001
Y × N × M	9	10.967	<0.0001	9	1.760	<0.0001
Position (P)	1	0.032	0.7423	1	0.776	0.0950
Y × P	1	0.201	0.4106	1	0.062	0.6369
N × P	3	1.376	0.0031	3	0.488	0.1540
Y × N × P	3	0.527	0.1497	3	0.444	0.1882
M × P	4	1.036	0.0075	4	1.315	0.0009
Y × M × P	3	0.627	0.0964	3	0.364	0.2693
N × M × P	12	0.268	0.5436	10	0.414	0.1377
Y × N × M × P	9	0.481	0.1038	5	0.319	0.3324
Error	1906	0.297		507	0.277	
Corrected Total	1977			572		

[y] All the data from all the sampling locations were taken into account.

[z] Data from sampling locations where no infected plant material was observed were disregarded.

Table 3. The average number of *B. cinerea* colony forming units (cfu's) observed on spore traps during 2003 and 2004 as calculated with analysis A[y]

	Position[z]	
Month	**Inside**	**Outside**
March	0.44 c	0.59 bc
April	1.42 a	1.47 a
May	1.39 a	1.30 a
June	0.58 bc	0.48 bc
July	0.48 bc	0.66 b
LSD	0.173	

[y] All the data from all the sampling locations were taken into account.

[z] Means indicated by different letters differed significantly at the 5% level according to the Student's t–least significant difference (LSD) test.

Table 4. The average number of *B. cinerea* colony forming units (cfu's) observed on spore traps during 2003 and 2004 as calculated with analysis B[y]

	Position[z]	
Month	**Inside**	**Outside**
March	0.48 c	0.59 c
April	1.23 ab	1.47 a
May	1.30 a	1.30 a
June	1.00 b	0.48 c
July	0.41 c	0.66 c
LSD	0.266	

[y] Data from sampling locations where no infected plant material was observed were disregarded.

[z] Means indicated by different letters differed significantly at the 5% level according to the Student's t–least significant difference (LSD) test.

Table 5. Analysis of variance on the mean[z] number of diseased seedling patches as represented by LNDP (data log transformed) in nurseries

Source of Variation	**Df**	**MS**	**P**
Year (Y)	1	3.054	<0.0001
Nursery (N)	3	4.035	<0.0001
Y × N	3	1.195	<0.0001
Month (M)	4	2.463	<0.0001
Y × M	3	1.499	<0.0001
N × M	12	1.353	<0.0001
Y × N × M	9	0.581	<0.0001
Error	1716	0.014	
Corrected Total	1751		

[z] Average values of data recorded over two seasons in four rooibos nurseries.

Table 6. Mean number of diseased rooibos seedling patches as represented by LNDP (data log transformed)[x] in four rooibos nurseries during 2003 and 2004

	Nursery[y]							
	A		B		C		D	
Month	**2003**	**2004**	**2003**	**2004**	**2003**	**2004**	**2003**	**2004**
March	–[z]	0.00 g	–	0.00 g	–	0.00 g	–	0.00 g
April	0.00 g	0.00 g	0.00 g	0.00 g	0.00 g	0.00 g	0.00 g	0.00 g
May	0.00 g	0.00 g	0.00 g	0.00 g	0.00 g	0.05 f (0.13)	0.00 g	0.00 g
June	0.00 g	0.36 c (1.28)	0.01 g (0.01)	0.00 g	0.12 e (0.31)	0.70 b (3.97)	0.00 g	0.00 g
July	0.00 g	0.16 d (0.46)	0.01 g (0.01)	0.00 g	0.13 e (0.33)	0.75 a (4.68)	0.00 g	0.00 g
LSD	0.049							

[x] Back-transformed data are given in brackets.

[y] Means indicated by different letters differed significantly at the 5% level according to the Student's t–least significant difference (LSD) test.

[z] No data. Sampling in 2003 only started in April.

Table 7. Analysis of variance on the mean incidence of plant material (asymptomatic rooibos seedlings, organic debris, weeds, rooibos cotyledons & vegetation) yielding *Botrytis cinerea* in and around rooibos nurseries from April to July in 2003 and from March to July in 2004

Source of Variation	**df**	**MS**	**P**
Year (Y)	1	2916.672	<0.0001
Nursery (N)	3	4155.580	<0.0001
Y × N	3	3162.024	<0.0001
Month (M)	4	1469.566	<0.0001
Y × M	3	875.395	<0.0001
N × M	12	913.074	<0.0001
Y × N × M	9	566.031	<0.0001
Position (P)	1	80320.0325	<0.0001
Y × P	1	304.290	0.0257
N × P	3	618.986	<0.0001
Y × N × P	3	179.922	0.0317
M × P	4	1705.485	<0.0001
Y × M × P	3	2642.207	<0.0001
N × M × P	12	329.382	<0.0001
Y × N × M × P	9	669.108	<0.0001
Error	1829	61.038	
Corrected Total	1900		

Table 8. The mean incidence of plant material (asymptomatic rooibos seedlings, organic debris, weeds, rooibos cotyledons and vegetation) yielding *B. cinerea* in and around four rooibos nurseries during 2003 and 2004

		Nursery[z]							
		A		**B**		**C**		**D**	
Year	**Month**	**Inside**	**Outside**	**Inside**	**Outside**	**Inside**	**Outside**	**Inside**	**Outside**
2003	April	1.80 s–u	18.75 j–n	1.84 s–u	20.83 i–l	3.61 r–u	53.33 a	0.39 u	25.00 f–j
	May	0.20 u	27.35 e–i	0.93 s–u	20.00 i–m	1.40 s–u	30.67 c–h	0.00 u	23.08 g–k
	June	0.00 u	44.23 b	0.20 u	20.00 i–m	0.00 u	38.54 bc	0.21 u	9.17 o–s
	July	1.67 s–u	8.33 o–t	0.80 u	19.58 i–m	0.22 u	17.50 i–n	0.26 u	14.82 l–p
2004	March	0.00 u	4.55 q–u	0.40 u	4.76 q–u	0.00 u	15.16 j–p	0.00 u	7.52 o–u
	April	3.28 r–u	31.30 c–g	0.35 u	11.18 n–r	0.70 u	30.56 c–h	0.90 tu	22.63 h–l
	May	25.08 f–j	32.07 c–f	0.20 u	29.44 d–h	2.13 s–u	36.12 b–d	0.00 u	17.21 j–o
	June	11.86 l–q	37.92 bc	0.00 u	5.13 q–u	2.07 s–u	34.39 c–e	0.17 u	38.51 bc
	July	5.61 q–u	60.89 a	0.00 u	53.33 a	0.33 u	31.04 c–g	0.60 u	27.22 b–d
LSD		8.381							

[z] Means indicated by different letters differed significantly at the 5% level according to the Student's t-least significant difference (LSD) test.

Figure 1. An example of the iron rods and petri dishes used as spore traps inside and around four rooibos nurseries.

3. RESISTANCE IN *BOTRYTIS CINEREA* TO IPRODIONE AS AN AID IN MONITORING INOCULUM DISPERSAL

ABSTRACT

Resistance of *Botrytis cinerea*, the causal agent of grey mould on rooibos seedlings, to iprodione has been documented in nurseries. Since resistance to dicarboximide fungicides is a genetically stable trait in *B. cinerea*, it has the potential to be used as a phenotypic marker to gain knowledge on the dispersal of *B. cinerea* inoculum inside and outside rooibos nurseries. Isolates of *B. cinerea* were collected from the air and from plant material in and around four rooibos nurseries in the Clanwilliam-area, Western Cape province, during 2003 and 2004. The isolates were assessed for resistance to iprodione at 1 and 3 µg/ml a.i. Isolates showed resistance to iprodione at 1 µg/ml a.i., but not at 3 µg/ml a.i. The initial incidence of dicarboximide-resistance at the four nurseries was slightly higher than would have been expected. As the season progressed, the incidence of iprodione-resistant isolates decreased towards May, after which an increase was observed towards July. Surprisingly, a relatively high percentage of isolates collected outside the nurseries was found to be dicarboximide resistant. Two of the nurseries had a significant higher incidence of resistant isolates on plant material collected inside, than on plant material collected outside the nursery. However, when looking at resistance levels of airborne isolates, no significant differences were found in the incidence of resistant isolates sampled inside and outside the four nurseries. The data indicated the importance of organic debris and seed-borne infections in the survival and dispersal of especially dicarboximide-resistant isolates of the pathogen.

INTRODUCTION

Rooibos [*Aspalathus linearis* (Burm. F.) R. Dahlg.] is a leguminous perennial of the bean family that occurs naturally in the Cedarberg Mountains of the Western Cape province. The most important foliar disease of rooibos seedlings, which can cause losses of up to 80% in some seasons if management practices are not implemented properly, is grey mould, caused by *Botrytis cinerea* Pers. ex Pers. (Lamprecht, 1996). The pathogen usually attacks the lower parts of the stem and foliage, causing wilt-like symptoms and occasionally death.

Botrytis cinerea is notorious for its ability to develop resistance towards new fungicides

in a relatively short period. Resistance towards dicarboximides in isolates of *B. cinerea* was reported across Europe only three to four years after introduction of these fungicides (Pommer & Lorenz, 1982; Steel & Nair, 1993). Subsequently, dicarboximide-resistant isolates of the pathogen have been reported on many crops all over the world (Steel & Nair, 1993; Russel, 1995). In South Africa, moderate resistance towards iprodione (dicarboximide) in *B. cinerea* isolates from rooibos seedlings were first observed in 1995 by Lamprecht *et al.* (1999).

Resistance to dicarboximide fungicides is thought to have evolved through at least three different *de novo* mutations in field populations (Cui *et al.*, 2004). This form of resistance development is thought not to occur as readily in the field as under laboratory conditions (Pommer & Lorenz, 1982). Regardless of the initial number of resistant isolates, the incidence in resistant isolates in the field can increase dramatically, reaching values of 90–100% after only 1 or 2 dicarboximide fungicide applications (Löcher *et al*., 1987; Vali & Moorman, 1992). In the absence of dicarboximide applications, the incidence of dicarboximide-resistant isolates of *B. cinerea* usually drops to low levels (approximately 10–20%) (Löcher *et al*., 1987). Dicarboximide-resistance appears to be genetically stable in *B. cinerea* (Wang *et al*., 1986; Vali & Moorman, 1992). Therefore, a decrease in the frequency of resistance can be ascribed to the lowered fitness of resistant strains in comparison to sensitive ones. The reduction in fitness seems to be inversely correlated with the level of resistance of the individual isolates. Isolates with a low-level of resistance (Beever *et al*., 1989; Vali & Moorman, 1992) and even moderately resistant *Botrytis* isolates, can exhibit fitness levels comparable to that of sensitive isolates, while highly resistant strains exhibited reduced fitness (Pommer & Lorenz, 1982). Conflicting reports exist on the nature of the reduction in fitness. Decreased sporulation of resistant isolates, for instance, has been observed by Pommer and Lorenz (1982) while Vali and Moorman (1992) did not detect any effect on sporulation. Several other reports indicated factors such as lowered infective ability (Pak *et al.*, 1990), mycelial growth rate (Pommer & Lorenz, 1982), virulence and production of sclerotia (Vali & Moorman 1992) to be involved in the reduced fitness of resistant isolates. These effects are all likely to differ between different environments and levels of resistance (Vali & Moorman, 1992).

Limited information exists on the inoculum sources of *B. cinerea* in rooibos nurseries. Although a previous study (Part 2) gave some indications on possible *B. cinerea* primary inoculum sources, no clear dispersal patterns could be discerned. The aim of this study was to gain information on the movement of dicarboximide-resistant strains of the pathogen in and outside rooibos nurseries. This should be possible since dicarboximide-resistance is a genetically stable trait in *B. cinerea*, making it suitable for use as a phenotypic marker.

MATERIALS AND METHODS

Isolates. Isolates of *B. cinerea* obtained during the 2003 and 2004 seasons (March – July) from the Clanwilliam area, Western Cape province, as part of a previous study (Part 2) were used. These isolates were collected from the air and plant material inside and around four rooibos nurseries (Table 1). The nurseries were subjected to weekly applications of fungicides to minimise losses due to disease (Table 2). Pure cultures of all these isolates were maintained on MEA (malt extract agar) slants at 4°C.

Fungicide sensitivity test. A stock solution of 1 µg/ml a.i. iprodione was prepared by adding 0.392 ml Rovral Flo (Rovral Flo 25 SC, Bayer) to 99.608 ml de-ionised water. Potato dextrose agar (PDA) (Biolab), cooled to 50ºC, was amended with 0, 1 and 3 ml of the stock solution to prepare media containing 0, 1 and 3 µg/ml a.i. iprodione. The amended media was poured into large Petri dishes (90 mm dia.). Freshly amended media was used during the entire assay.

Mycelial plugs from agar slants were sub-cultured onto PDA (200 g sliced potatoes autoclaved in one liter de-ionised water, strained, 20 g dextrose and 12 g agar added and autoclaved) and incubated for 3–4 days at 22°C. For each isolate five plugs (5 mm dia.) from the edge of an actively growing culture were each placed upside down on a Petri dish containing PDA amended with 0 (one dish), 1 (two dishes) or 3 (two dishes) µg/ml a.i. iprodione. Ten different isolates were plated per Petri dish, with nine plugs evenly spaced in a clockwise order around the perimeter of the dish and the tenth plug being placed in the middle of the dish. The presence or absence of fungal growth was recorded after incubation at 22°C for 36 hours. An isolate was designated resistant if it grew on the control plate and fungicide-amended plates, and sensitive if it grew only on the control plate. The incidences of resistant isolates were calculated for each treatment.

Statistical analysis. The survey consisted of a 4 × 5 × 2 × 2 factorial with four nurseries (A, B, C, D), five months (March – July), two positions (inside, outside) and two sources (air, plant material). The two seasons during which the survey was conducted (2003, 2004) served as repetitions. The percentage incidence of resistance was transformed to logits. Both the

percentage and logit values were subjected to a factorial analysis using SAS V8.2 (SAS, 1999). Shapiro-Wilk's test was performed to test for non-normality (Shapiro & Wilk, 1965). The Student's t–LSD was calculated at a 5% significance level to compare means of significant interactions or main effects. Deviations from normality did occur, but since such differences could be ascribed to kurtosis the results were deemed suitable for interpretation (Glass *et al.*, 1972).

RESULTS

Several isolates of *B. cinerea* exhibited reduced sensitivity to iprodione at a concentration of 1 µg/ml, but no resistance was found at the higher concentration (3 µg/ml). The data presented below is therefore only applicable to isolates resistant to iprodione at 1 µg/ml. The utilisation of the two seasons as repetitions prevented the inclusion of this factor in the interactions observed during the analysis of variance (ANOVA). The results obtained with the logit transformed data did not differ from the original percentage incidence values and will therefore not be discussed.

The ANOVA for the influence of year, nursery, month, position and source on the percentage incidence of iprodione resistance indicated a significant interaction ($P < 0.0001$) between the different nurseries, positions and sources (Table 3). The ANOVA also indicated months ($P = 0.0453$) and years ($P < 0.0001$) to be significant main effects (Table 3).

Differences were observed in the incidence of resistant isolates. On plant material, the incidence of resistant isolates at nurseries A and B was significantly higher ($P = 0.05$) inside than outside the nurseries (Table 4). There was, furthermore, also a significantly higher incidence of resistant isolates on plant material than in airborne samples inside nurseries A and B (Table 4). However, when the incidence of resistance was determined in airborne samples, there was no significant difference between any of the nurseries, whether isolates were collected inside or outside the nursery (Table 4).

The mean incidence of iprodione-resistant *B. cinerea* isolates in the air and plant material varied during the growing season. The initial incidence of dicarboximide-resistance in the four nurseries was slightly higher than would have been expected. Surprisingly, a relative high percentage of isolates collected outside the nursery was found to be dicarboximide-resistant. The incidence of iprodione-resistant isolates decreased slightly from March to May and

increased again towards July (Figure 1).

DISCUSSION

A relative high incidence of dicarboximide-resistant isolates was observed on vegetation outside the nurseries. It seems unlikely that such a high incidence of resistance should be present in isolates from vegetation outside the nurseries, since these populations would not have been exposed to the selection pressure exerted by fungicide applications. Furthermore, it is generally accepted that resistance in *B. cinerea* to dicarboximides is associated with some degree of reduced fitness when compared to sensitive isolates (Davis & Dennis, 1981; Löcher *et al.*, 1987; Latorre *et al.*, 1994; Cui *et al.*, 2002). However, rooibos nurseries are often located next to sites previously used as nurseries where *B. cinerea* populations have been exposed to several fungicide sprays including iprodione and captab. Therefore, these *B. cinerea* populations could possibly survive on vegetation and organic debris and may explain the high levels of initial resistance outside some nurseries. Studies in Israel have demonstrated the survival of dicarboximide-resistant isolates during hot, dry summers for up to 8 months (Katan, 1982; Yunis & Elad, 1989) similar to those of the rooibos production areas. Furthermore, frequent applications of captab (Captab 50 SC, Dow AgroSciences) and chlorothalonil (Bravo 50 SC, Effekto) in nurseries at the beginning of each season might have influenced these observations, since incomplete cross-resistance between iprodione and fungicides of the phthalimide group (such as captab and folpet) have been recorded (Barak & Edgington, 1984; Fourie & Holz, 2001). Cross-resistance of *B. cinerea* to chlorothalonil (another fungicide sprayed in rooibos nurseries) and iprodione has also been demonstrated (Barak & Edgington, 1984). Isolates of *B. cinerea* with resistance to captab have levels of pathogenicity similar to that of sensitive isolates (Barak & Edgington, 1984). Consequently the persistence of such isolates in mixed populations of the pathogen is not unlikely.

Löcher *et al.* (1987) suggested that several factors could be involved in changes in the incidence of dicarboximide-resistance, besides the application of fungicides. These included climatic conditions, the type and number of treatments and infection pressure. Milgroom (1990) also stressed the importance of pathogen population sizes in the development of fungicide resistance. Since the climatic conditions and population sizes of *B. cinerea* differed between the four nurseries (as was shown in Part 2), differences in the incidence of resistance observed between the nurseries are not unexpected (Table 3). The fact that these differences were mainly observed on isolates of *B. cinerea* collected from plant material inside the nurseries indicates that

especially the infection pressure and differences between cultural practices are of importance in the development of resistance to iprodione in rooibos nurseries.

Positional differences between the incidences of dicarboximide-resistant isolates would also be expected since the application of iprodione inside the nurseries would select the resistant sub-population for survival, thereby increasing the incidence of iprodione-resistant isolates. This was evident in nurseries A and B where large differences were observed between the incidences of iprodione-resistant isolates from plant material inside and outside the nurseries. In nursery A the continuous applications of iprodione might have resulted in these high levels of fungicide resistance. Besides the selection pressure exerted by iprodione applications, cross-resistance to other fungicides applied in the nurseries might also have played a role in the development of these high incidences of resistance. Other instances of the sudden increase of dicarboximide resistance to such high levels have also been recorded (Löcher *et al.*, 1987; Vali & Moorman, 1992). Contrarily, in nurseries C and D, the incidences of resistant isolates from plant material inside and outside the nurseries did not differ, even though iprodione was applied in the nurseries. In these nurseries iprodione applications were alternated with pyrimethanil on a weekly basis. Several reports have indicated a lack of cross-resistance between dicarboximide fungicides and pyrimethanil or related fungicides (anilinopyrimidines) (Forster & Staub, 1996; Petsikos-Panayotarou *et al.*, 2003). The lack of increase of iprodione-resistant isolates inside these nurseries could therefore be a result of this alternation with pyrimethanil. The high number of iprodione-resistant isolates observed inside nursery B, despite the alternation of iprodione and pyrimethanil could have been caused by the timing of sampling in relation to the application of the fungicides. Sampling after iprodione applications could have resulted in the collection of more iprodione-resistant isolates, since the selection pressure exerted by the fungicide would have favoured the proliferation of this part of the pathogen population.

The incidence of resistance in *B. cinerea* populations collected from the air did not differ between nurseries, whether populations were collected inside or outside the nurseries (Table 3). This observation confirms previous observations (Part 2) that most *B. cinerea* colonies developing on spore traps originated from non-specific sources not located within the area sampled ("background contamination"). This is also substantiated by higher incidences of resistant isolates in the air than on plant material inside nursery C and outside nurseries B and D. Even in cases where high incidences of resistance in *B. cinerea* occurred on plant material inside the nurseries (nurseries A and B) the levels of airborne resistant isolates inside the nurseries did not increase. This may be ascribed to the decreased sporulation potential of dicarboximide-resistant *B. cinerea* (Davis & Dennis, 1981) as well as the prevalence of "background

contamination" on the spore traps. The few conidia that would be produced would be expected to have a limited dispersal ability (Johnson & Powelson, 1983) and apparently do not escape the seedling canopies. This phenomenon stands in contrast to what was observed in a previous study (Part 2) where a high incidence of grey mould in the nurseries was reflected in higher incidence of *B. cinerea* colonies observed on spore traps inside the nurseries. The conidia that gave rise to the development of these colonies could therefore be assumed to have originated from dicarboximide-sensitive isolates.

Although differences in the incidence of *B. cinerea* isolates exhibiting resistance toward iprodione were observed through the growing season, these differences were only slight (Figure 1). In fact, the only significant difference was observed between May and July. The initial presence of dicarboximide-resistant isolates seems to corroborate other reports concerning the 'oversummering' of strains of *B. cinerea* resistant to dicarboximides (Katan, 1982; Yunis & Elad, 1989). The initial value of 21.94% is slightly higher than the base level values (10—20%) reported in grapevines at the start of the season (Löcher *et al.*, 1987). Applications of captab and chlorothalonil might also have had an influence in establishing this early incidence of resistance. As the season progressed, the incidence of resistance decreased toward May. The progression of the seasons from summer to autumn during this part of the year would favour the proliferation of *B. cinerea*. Under such conditions differences in fitness between dicarboximide-resistant and -sensitive isolates would gain importance, since the increase in activity and growth of *B. cinerea* would lead to competition for tissues suitable for colonisation (Löcher *et al.*, 1987). This would result in a decrease in the percentage incidence of resistant isolates. From May onwards, the incidence of iprodione-resistance increased towards a maximum of 23.20% in July. Since iprodione applications inside the nurseries are usually made from the end of May to the end of the season, this increase could be due to increased selection pressure exerted on the pathogen population.

Due to the complexity of the factors (and their interactions) involved in the development and establishment of dicarboximide-resistance in rooibos nurseries the initial aims of this study (the elucidation of the sources and dispersal mechanisms of primary inoculum of *B. cinerea*) could not be fully realised. A few deductions can, however, be made concerning the dispersal and survival of iprodione-resistant isolates of the pathogen in rooibos nurseries. Very little living plant material was present for sampling in the nurseries during March 2004 due to sanitation practices implemented during the preparation of the seedbeds and seedlings that had not yet emerged from the soil. Despite the fact that the local dispersal of dicarboximide-resistant isolates through conidia is very limited such isolates were present on plant material inside the

nurseries in April of the same year. Since the development of fungicide resistance is dependent on the presence of resistant isolates (aside from *de novo* mutational development of resistance), it would therefore seem as if seeds and small pieces of organic debris are an important source of dicarboximide-resistant isolates. These materials could either be dispersed into the nursery from infected sources outside the nursery through the action of humans, insects or wind or they could be present in and on the nursery soil at the start of the season. Another possibility is the introduction of airborne resistant isolates from the "background contamination" observed on the spore traps.

These results support hypotheses made previously (Part 2) concerning the dispersal and survival of *B. cinerea* through small pieces of infected organic material and/or infected seeds of weeds. These materials are especially of importance as vectors of dicarboximide-resistant isolates of the pathogen, since these would be expected to have lowered sporulation ability.

Future research regarding the inoculum ecology and dispersal of fungicide-resistant *B. cinerea* should be performed to cast more light on this subject. Such studies would need to be conducted under more controlled conditions in order to discern the effects of different factors involved in the development, survival and dispersal of resistant isolates.

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Table 1. Numbers of isolates tested for resistance to iprodione

Source	Nursery							
	A		B		C		D	
	Inside	Outside	Inside	Outside	Inside	Outside	Inside	Outside
Air	63	61	55	48	69	64	62	64
Plant material	223	99	11	37	156	224	11	171

Table 2. Program for the application of fungicides in rooibos nurseries as recommended by Rooibos Ltd.

Application (days after sowing)	**Fungicides**[y]
7 (1 week)	captab
10 days	captab (drench)
14 (2 weeks)	captab
21 (3 weeks)	captab
24 days	captab (drench)
28 (4 weeks)	captab & iprodione
35 (5 weeks)	captab
42 (6 weeks)	captab & chlorothalonil
49 (7 weeks)	captab & chlorothalonil
56 (8 weeks)	captab & chlorothalonil
63 (9 weeks)	captab & chlorothalonil
70 (10 weeks)	captab & chlorothalonil
77 (11 weeks)	pyrimethanil (or iprodione)[z]
84 (12 weeks)	chlorothalonil & iprodione
91 (13 weeks)	pyrimethanil (or iprodione)
98 (14 weeks)	chlorothalonil & iprodione
105 (15 weeks)	pyrimethanil (or iprodione)
112 (16 weeks)	chlorothalonil & iprodione

[y] captab = Captab (Dow Agrosciences) at 0.5g a.i./0.5l/m^2; captab (drench) at 2g a.i./2l/m^2; chlorothalonil = Bravo (Effekto) at 0.5ml a.i./0.5l/m2; iprodione = Rovral Flo (Bayer) at 0.25ml a.i./0.5l/m^2; pyrimethanil = Scala (Bayer) at 0.4 g a.i./0.5l/m^2

[z] Pyrimethanil applications were substituted for iprodione applications in nursery A.

Table 3. Analysis of variance on the percentage incidence of iprodione-resistant (1 g/ml) isolates of *Botrytis cinerea* collected in- and outside rooibos nurseries

Source of Variation	**Df**	**MS**	**P**
Year (Y)	1	15072.617	<0.0001
Nursery (N)	3	1459.661	0.0006
Month (M)	4	563.233	0.0453
N × M	12	262.494	0.2915
Position (P)	1	1403.395	0.0136
N × P	3	1217.732	0.0020
M × P	4	220.913	0.3989
N × M × P	12	298.346	0.2003
Source (S)	1	1368.509	0.0147
N × S	3	2059.432	<0.0001
M × S	4	232.350	0.3727
N × M × S	12	143.353	0.7688
P × S	1	4204.675	<0.0001
N × P × S	3	2700.730	<0.0001
M × P × S	4	301.304	0.2445
N × M × P × S	9	204.135	0.4867
Error	47	213.309	
Corrected Total	124		

Table 4. Percentage incidence of *B. cinerea* isolates resistant to iprodione in- and outside four rooibos nurseries during 2003 and 2004

Source	Nursery[y]							
	A		B		C		D	
	Inside	Outside	Inside	Outside	Inside	Outside	Inside	Outside
Air	15.49 cde	14.90 cde	15.48 cde	19.14 cde	18.36 cde	11.17 de	12.19 de	22.84 cd
Plant material	47.89 b	27.62 c	82.67 a	15.42 cde	4.97 e	17.50 cde	15.28 cde	10.16 de
LSD	15.175							

[y] Values indicated by different letters differed significantly at the 5% level according to the Student's t-least significant difference (LSD) test.

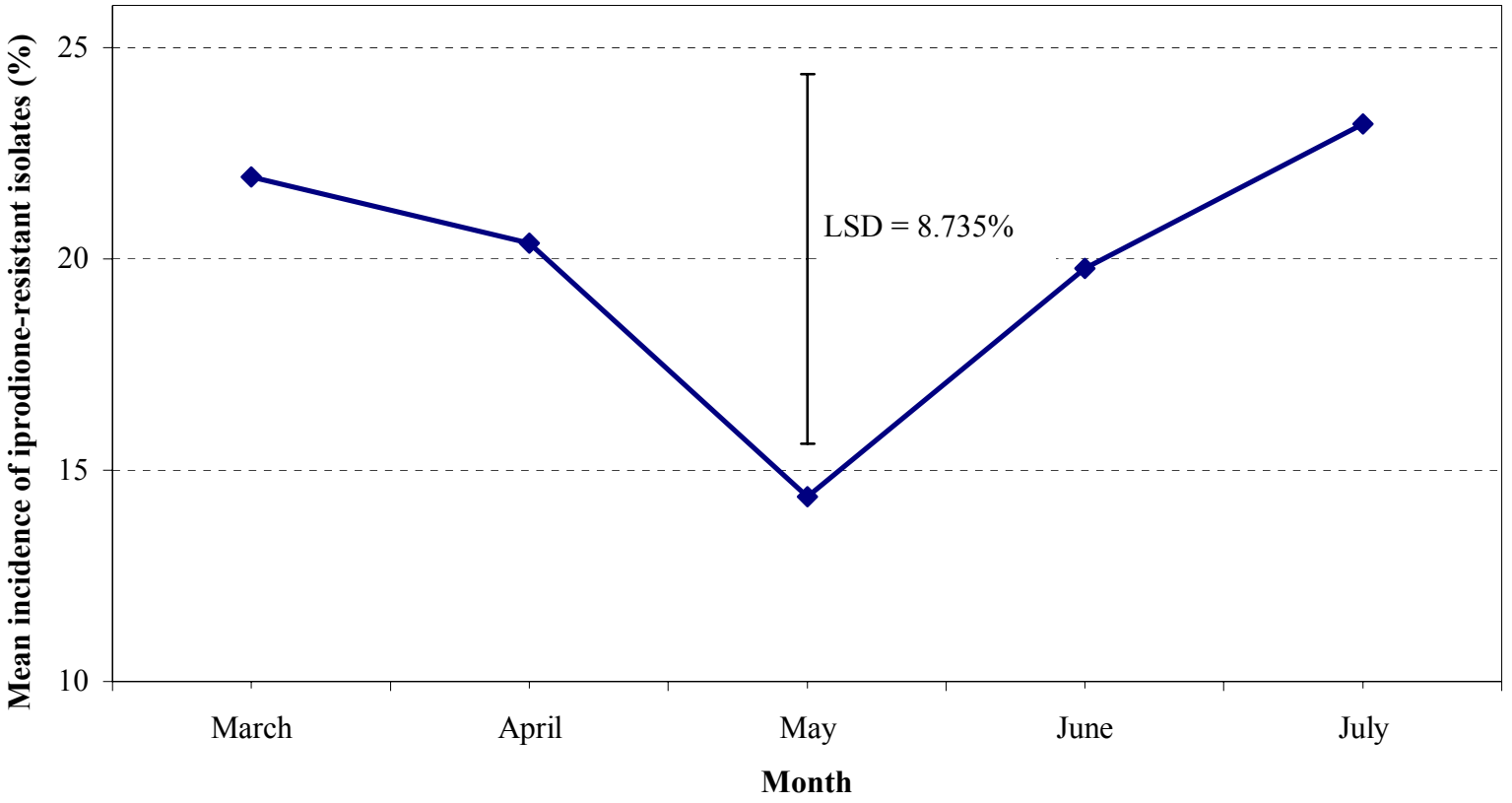

Figure 1. The mean incidence of *B. cinerea* isolates resistant to iprodione at 1 µg/ml in four rooibos nurseries during two production seasons (2003, 2004).